

Stem Cells and Metabolism Research Program, Faculty of Medicine, University of Helsinki and Doctoral Programme in Biomedicine, University of Helsinki

# **MECHANISMS AND DYNAMICS OF MITOCHONDRIAL DISEASE STRESS RESPONSES: SPECIAL EMPHASIS ON FGF21**

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ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Medicine of the University of Helsinki, for public examination in Lecture Room 1, Biomedicum Helsinki, on 6 March 2020, at 12 noon.

Helsinki 2020

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Cover graphics: Hippopotamus made of autoradiography images of mouse  
hippocampus.

Dissertationes Scholae Doctoralis Ad Sanitatem Investigandam Universitatis  
Helsinkiensis

ISBN 978-951-51-5860-4 (print)

ISBN 978-951-51-5861-1 (online)

ISSN 2342-3161 (print)

ISSN 2342-317X (online)

Unigrafia, Helsinki 2020

*“Before I came here I was confused about this subject. After listening to your lecture, I am still confused but at a higher level.”*

*–Enrico Fermi*

# ABSTRACT

Dominant mutations in mitochondrial replicative helicase TWINKLE are a common cause of autosomal dominant progressive external ophthalmoplegia (AdPEO), a mitochondrial myopathy characterized by accumulation of multiple mtDNA deletions and respiratory chain deficiency in muscle, heart and central nervous system. Our research group has previously generated a transgenic mouse, Deletor, that replicates the main pathological characteristics of the AdPEO-disease. Studies on the Deletor have revealed induction of local stress response in the muscle that involved expression and secretion of a metabolic hormone, Fibroblast growth factor 21 (FGF21). In physiological nutritional challenges, FGF21 is known to be secreted by the liver to enhance energy-metabolism. Therefore, high levels of circulating FGF21 in mitochondrial disease expose the whole body to non-homeostatic, chronically altered metabolic state.

The overall aim of this thesis was to characterize the stress responses and metabolic rearrangements upon mitochondrial myopathy with special emphasis on the actions of FGF21. We utilized valuable biopsy and serum samples of patients in parallel analyses with the Deletor and other established mouse models, and generated a new Deletor-FGF21 knockout model.

First, to characterize the metabolic status of the affected muscle tissue, we utilized mass-spectrometric metabolomics and transcriptional analyses. Overall, the metabolic signature of the muscle was conserved in AdPEO patients and Deletor including rearrangement of one-carbon, folate and nucleotide metabolism. Moreover, the muscle and heart of the Deletor presented with robust induction of glucose-driven serine synthesis and glutathione production, demonstrated with *in vivo* radiotracer and flux-omics assays. Together, these novel metabolic rearrangements and the previously characterized transcriptional stress response are hereafter collectively called the mammalian mitochondrial integrated stress response, or ISR<sup>mt</sup>.

Next, we studied the dynamics of the pathological events in the muscle by analyzing ISR<sup>mt</sup> markers at different stages of manifestation in the Deletor. At the time when the first histological signs of mitochondrial dysfunction are detected (<12 months of age) the mitochondrial folate cycle and endocrine hormones FGF21 and GDF15 were induced. Several months later, when some of the affected muscle fibers already manifest with pathological mitochondrial proliferation, we detected induction of serine *de novo* synthesis enzymes, marking initiation for the glucose driven metabolic rearrangements. Importantly, characterization of muscle pathology of the Deletor-FGF21KO model revealed that FGF21 drives the progression of ISR<sup>mt</sup> to the advanced metabolic stage, whereas the molecular and histological disease hallmarks, or expression of the first-stage ISR<sup>mt</sup> markers, were not affected by presence or absence of FGF21.

Systematic phenotypic characterization of the Deletor-FGF21KO showed that FGF21 expectedly advanced white adipose tissue browning and loss of systemic adiposity in the Deletor. Moreover, we demonstrated that tissue-level glucose preferences were altered in the whole brain and peripheral organs of Deletor by FGF21. Additionally, we described a completely new central nervous system pathology in the Deletor. Importantly, we showed that, although the mtDNA deletion formation is uniform in the brain of Deletor, only a specific CA2-region of hippocampus manifests with mitochondrial respiratory chain pathology combined with intensive glucose uptake.

The second part of this thesis involved clinical characterization of the circulating biomarkers of mitochondrial diseases, FGF21 and GDF15. In our combined meta-analysis and retrospective measurements, we confirmed that both FGF21 and GDF15 outperform the traditional metabolite markers of mitochondrial disease. Interestingly, however, among the mitochondrial myopathy patients and representative mouse models, we found that high levels of serum FGF21 and GDF15 were characteristic for mitochondrial myopathies caused by primary or secondary mitochondrial translation defects, whereas primary OXPHOS mutations did not induce the same response, important to acknowledge in diagnostics and mechanistic studies in the future.

In summary, studies of this thesis present for the first time the conserved whole-cellular metabolic rearrangements of the muscle upon mtDNA maintenance defects. We also present indispensable roles for FGF21 in regulation of local and systemic disease progression in a physiological model of mitochondrial myopathy. Together, our pre-clinical and clinical analyses on the stress responses upon different mitochondrial insults have unlocked novel research directions and serve the mitochondrial disease diagnostics, follow-up and evaluation of treatment trials in the future.

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# LIST OF ORIGINAL PUBLICATIONS

This thesis is officially (\*) based on the following publications (I and II), referred in the text by their roman numerals.

- I      **Forsström S.** <sup>+</sup>, Jackson C.B. <sup>+</sup>, Carroll C.J., Kuronen M., Pirinen E., Pradhan S., Marmyleva A., Auranen M., Kleine I., Khan N.A., Roivainen A., Marjamäki P., Liljenbäck H., Wang L., Battersby B.J., Richter U., Velagapudi V., Nikkanen J., Euro L., Suomalainen A. Fibroblast Growth Factor 21 Drives Dynamics of Local and Systemic Stress Responses in Mitochondrial Myopathy with mtDNA Deletions. *Cell Metabolism* (2019) Dec 3;30(6):1040-1054.e7
- II      Lehtonen J.M. <sup>+</sup>, **Forsström S.** <sup>+</sup>, Bottani E., Viscomi C., Baris O.R., Isoniemi H., Höckerstedt K., Österlund P., Hurme M., Jylhävä J., Leppä S., Markkula R., Heliö T., Mombelli G., Uusimaa J., Laaksonen R., Laaksovirta H., Auranen M., Zeviani M., Smeitink J., Wiesner R.J., Nakada K., Isohanni P., Suomalainen A. FGF21 is a biomarker for mitochondrial translation and mtDNA maintenance disorders. *Neurology* (2016) Nov 29;87(22):2290-2299.

(<sup>+</sup>) Shared contribution. Publication II was discussed also in the academic dissertation by J.M. Lehtonen (2017).

(\*) In this thesis, data from an unlisted original publication by Forsström S. is presented and discussed. Due to regulations on the number of shared contents in dissertations by the Faculty of Medicine, this original publication was not included to the publication list. The third publication is referred in the text as Publication III\*, or III\*. Full reference:

Nikkanen J., **Forsström S.**, Euro L., Paetau I., Kohnz R. A., Wang L., Chilov D., Viinamäki J., Roivainen A., Marjamäki P., Liljenbäck H., Ahola S., Buzkova J., Terzioglu M., Khan N.A., Pirnes-Karhu S., Paetau A., Lönnqvist T., Sajantila A., Isohanni P., Tynismaa H., Nomura K., Battersby B.J., Velagapudi V., Carroll C.J., Suomalainen A. Mitochondrial DNA Replication Defects Disturb Cellular dNTP Pools and Remodel One-Carbon Metabolism. *Cell Metabolism* (2016), Apr 12;23(4):635-48.

# ABBREVIATIONS

<sup>1</sup> C	one-carbon
[ <sup>18</sup> F]-FDG	2- <sup>18</sup> Fluoro-2-deoxy-D-glucose
[ <sup>18</sup> F]-FTHA	14-(R,S)- <sup>18</sup> Fluoro-6-thia-heptadecanoic acid
AARE	amino acid response element (promoter sequence)
ADP/ATP	adenosine diphosphate/adenosine triphosphate
ATF	activating transcription factor
CBS	cystathionine beta synthase
CoA	coenzyme A
COX	cytochrome c oxidase
CTH	cystathionine gamma lyase
DEL	Deletor mouse
DHC	dorsal hippocampus
dNTP	deoxynucleotide triphosphate
FAD(H <sub>2</sub> )	flavin adenine dinucleotide (reduced)
FGF21	fibroblast growth factor 21
FGF21KO/FKO	FGF21 knockout
GCLC	glutamate-cysteine ligase
GDF15	growth and differentiation factor 15
HSP	heat shock protein
IOSCA	Infantile-onset spinocerebellar ataxia syndrome
ISR <sup>mt</sup>	(mammalian) mitochondrial integrated stress response
MELAS	Mitochondrial encephalopathy, lactic acidosis and stroke-like episodes
MERRF	Myoclonic epilepsy with ragged-red fibers
MIRAS	Mitochondrial recessive ataxia syndrome
MLASA	Myopathy with lactic acidosis
MNGIE	Mitochondrial neurogastrointestinal encephalopathy
mTORC1	mechanistic target of rapamycin complex 1
mRNA	messenger RNA
mtDNA	mitochondrial DNA
NAD(H)	nicotinamide adenine dinucleotide (reduced)
OXPHOS	oxidative phosphorylation
(ad)PEO	(autosomal dominant) Progressive external ophthalmoplegia
PET	positron emission tomography
PHGDH	phosphoglycerate dehydrogenase
PSAT1	phosphoserine aminotransferase 1
(q)PCR	(quantitative) polymerase chain reaction
rRNA	ribosomal RNA

ROS	reactive oxygen species
SDH	succinate dehydrogenase
TCA	tricarboxylic acid cycle (or citric acid or Krebs cycle)
THF	tetrahydrofolate
tRNA	transfer RNA
UPR <sup>mt</sup>	mitochondrial unfolded protein response

# 1 INTRODUCTION

Evolution of the eukaryotic cells is marked by compartmentalization of metabolism (Bolte, 2015). For example, synthesis and breakdown of fatty acids is separated between cytoplasm, mitochondria and peroxisomes, and regulated by shuttling of precursors and intermediates between the compartments. Some metabolic pathways are partitioned between cytoplasm and mitochondria to form complete functional metabolic cycles, e.g. the folate cycle. Perhaps the most famous function of mitochondria is to perform final oxidation of carbohydrates and fatty acids, followed by oxidative phosphorylation. Oxidative phosphorylation, or OXPHOS, is the most efficient way to produce the conserved energy currency of the cell, ATP, and simultaneously maintain redox-status of the cell.

Properly functioning OXPHOS of the eukaryotic cell needs coordinated expression of nuclear factors and the organellar genome of mitochondria, mtDNA. MtDNA is a circular genome that only encodes a handful of OXPHOS enzyme subunits and all RNA-molecules needed for in-organelle translation of mtDNA transcripts. Mutations in the mtDNA and its nuclear-encoded maintenance factors are the most frequent causes of mitochondrial diseases.

Mitochondrial diseases are a spectrum of disorders fundamentally characterized by impaired respiratory chain and OXPHOS, failures in which compromise energy metabolism and maintenance of the balanced oxidative environment for various enzymatic reactions. Symptoms and tissue-specific manifestations vary tremendously from one disorder to another – spanning from infertility and adult-onset myopathy to fatal seizures and metabolic crisis of an infant (Ylikallio, 2012). Mitochondrial diseases are known to be sensitive to environmental input, nutritional status and developmental state of an individual, and altered hormonal signaling could have a critical effect in the pathophysiology of a disease. Therefore, mitochondrial diseases are difficult to diagnose and after decades of research have still remained incurable. Characterization of local and systemic metabolic rearrangements, and factors that regulate those events, are key for understanding the mechanisms that allow better diagnostics, enable identification of new therapy targets and serve in evaluation of treatment trials in the future (Suomalainen, 2018).

In this thesis, we have investigated the stress responses in mitochondrial diseases *in vivo*.

## **2 REVIEW OF THE LITERATURE**

### **2.1 FUNDAMENTALS OF ENERGETICS IN MITOCHONDRIA**

#### **2.1.1 STRUCTURE, DYNAMICS AND DYNAMIC STRUCTURE**

Mitochondria are intracellular organelles with several specific characteristics: in the animal kingdom, they are the only organelles with their own DNA (Nass, 1965), protein synthesis apparatus (McLean JR, Cohn GL, Brandt IK, 1958; Wintersberger, 1964), and they are electrochemically charged (Mitchell, 1961). The main tasks of mitochondria involve a variety of biosynthetic functions from ATP synthesis and redox-regulation to synthesis of sulfur clusters and metal metabolism.

The origin of mitochondria is approximated to have taken place 1.5 billion years ago in an event of endosymbiosis (Sagan (née Margulis), 1967). According to a widely-accepted theory an anaerobic eukaryote engulfed a prokaryotic endosymbiont. It has also been suggested that an archaeon engulfed aerobic proteobacteria, and that event sparked the separation of the eukaryotes from the lineage of archaea (discussed in Lane 2017). Although speculations on the exact primitive benefit for the host from having an endosymbiont have not reached conclusion, the eukaryotic cells with mitochondria are the only ones that compose complex multicellular life-forms. According to bioenergetic calculations, the membrane surface area devoted for energy production in mitochondria enabled formation, maintenance and abundant expression of enormous nuclear genomes, and therefore led to emergence of the multigenic eukaryote-specific traits, e.g. cell cycle, sexual dimorphism, endomembrane trafficking and the nucleus (discussed in Lane and Martin 2010).

Structure and functions of the eukaryotic mitochondria still resemble closely the prokaryotic systems, reflecting their evolutionary ancestry. Mitochondria have two lipid bilayers, the outer and inner membranes which enclose two chemically distinct compartments, intermembrane space and mitochondrial matrix. The physical barriers formed by the two membranes maintain mitochondrial membrane potential, an electrochemical proton gradient across the inner membrane (Figure 2). Essentially, failure to maintain the mitochondrial membrane potential is penalized by collapsed energy production, transmembrane transport and cell death (Zorova, 2018). Structurally, the inner mitochondrial membrane is intensively folded to form cristae structures that protrude to the matrix. The cristae are extremely protein rich, occupied e.g. by OXPHOS complexes. The mitochondrial matrix is an active center of metabolism, occupied by essential enzymes of carbohydrate and fatty acid oxidation as well as tens of biosynthetic pathways.

The great majority of the approximately 1200 mitochondrially targeted proteins (Calvo, 2016) are nuclear encoded and synthesized in the cytoplasm. Those proteins, as well as most metabolism intermediates, need regulated transport through the outer and inner membranes, while some small molecules can freely diffuse in and out of mitochondria (Fox, 2012; Palmieri, 2014).

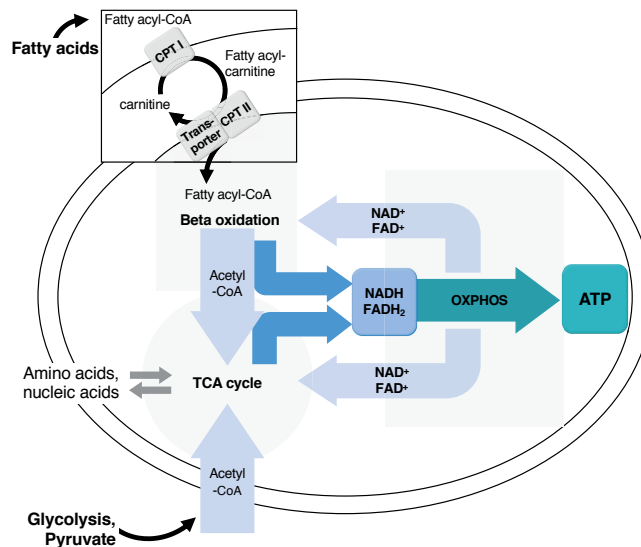
Mitochondria of one cell form extremely complex and dynamic networks dependent on cell type and metabolic status. Mitochondria undergo constant fission and fusion events that are carried out by distinct machineries in close contact with cytoskeleton and endoplasmic reticulum (Kuznetsov, 2013; Lewis, 2016; Pernas, 2016; Rieusset, 2018). Impairment of fission or fusion machineries cause optic atrophy and neuromuscular diseases (Delettre, 2002; Züchner, 2004). The dynamic movement and transportation of mitochondria is powered by kinesin and dynamin motor proteins along microtubule tracks, or along actin filaments by myosin motors (Hollenbeck, 2005). Proper transportation of mitochondria along polarized structures, such as axons of neurons, is important for cellular fitness (Ferreirinha, 2004), and has reported implications even in common neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases (Takahara, 2015; Zheng, 2019).

### **2.1.2 THE POWERHOUSE**

Metabolism is the sum of chemical transformations in a cell or an organism. Metabolic pathways are divided into catabolic and anabolic processes that respectively break down or synthesize organic macromolecules. Catabolic reactions convert energy-rich macronutrients to smaller end-products. In the oxidative reactions, electrons are released and the energy is conserved in the form of ATP or reduced electron carriers and cofactors. These high-energy products of oxidative catabolism are correspondingly consumed by anabolic biosynthesis.

Mitochondrial OXPHOS is the most productive and efficient machinery for ATP production in mammalian cells. Therefore, mitochondria have been called the powerhouse of the cell. However, maintenance of the correct ratios between the reduced and oxidized electron carriers is as important as maintaining levels of ATP, since numerous enzymatic reactions are regulated by the reductive state of the cell. (Nelson and Cox, 2008, part II, chapters 13-23).

The mitochondrial matrix hosts enzymatic machineries for final catabolism of carbohydrates and fatty acids, tricarboxylic acid cycle (TCA, also called citric acid or Krebs cycle) and beta oxidation, respectively. The main end-products of TCA cycle and beta oxidation are the reduced cofactors, nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH<sub>2</sub>), that are utilized by mitochondrial OXPHOS to 1) produce ATP and 2) retain the levels of oxidized cofactors NAD<sup>+</sup> and FAD<sup>+</sup> (Figure 1) (Nelson and Cox, 2008, chapter 13-14; Martínez-Reyes, 2016).



**Figure 1** Oxidative energy metabolism in mitochondria. Fatty acid beta oxidation and TCA cycle conserve energy from dietary fats and carbohydrates in form of reduced electron carriers, NADH and FADH<sub>2</sub>. Electron transport chain and OXPHOS generate ATP, and regenerate the oxidized cofactors NAD<sup>+</sup> and FAD<sup>+</sup> (see also Figure 2). Abbreviations: CPTI(II)= carnitine acyltransferase I(II).

### 2.1.2.1 TCA cycle is central in oxidative catabolism of carbohydrates and fatty acids

The TCA cycle oxidizes metabolic intermediates in cyclic reactions that release CO<sub>2</sub>. The oxidative reactions generate electrons that are conserved in NADH and FADH<sub>2</sub>. In addition, one molecule of ATP or GTP is produced per cycle. In oxidative nutrient rich conditions, acetyl coenzyme A (acetyl-CoA), is accepted as the primary substrate for the TCA cycle. The main producers of acetyl-CoA are fatty acid oxidation and glycolysis (Shi, 2015). Conversely, upon scarce nutrition or specific biosynthetic need, the TCA cycle can be utilized by the cell in intermediary metabolic reactions, allowing alternative entry and interconversion of TCA cycle metabolites, amino acids and nucleic acids (Boroughs, 2015) (Figure 1).

Glucose provides easily accessible energy for the cell. In nutrient rich conditions catabolism of dietary and storage carbohydrates dominate energy metabolism. Cytoplasmic glycolysis consists of preparatory and energy conserving phases. The preparatory phase consumes ATP and produces three-carbon intermediates for further catalysis or biosynthetic reactions. For example, glycolysis intermediates glyceraldehyde-3-phosphate (G3P) and 3-phosphoglycerate (3-PG) serve as precursors for membrane lipids and *de novo*

synthesis of non-essential amino acids, respectively (Vander Heiden, 2009). The energy conserving pay-off phase produces three-carbon metabolite pyruvate, and generates ATP and NADH. Pyruvate is transported to mitochondria and further oxidized into a two-carbon acetyl-CoA molecule for oxidation in the TCA cycle. (Nelson and Cox, 2008, chapter 14).

Fatty acids are breakdown products of dietary fats. Fatty acids are structurally variable with regard to the length of the carbon chain and presence and abundance of double bonds in the backbone (saturated vs. unsaturated fatty acids). For metabolic utilization (catabolic or anabolic), the free fatty acids are activated by addition of coenzyme A, yielding fatty acyl-CoA in reactions where ATP is consumed. The mitochondrial outer membrane is occupied by general acyl-CoA synthetases that have specificity for different lengths of fatty acids. To be catabolized in the mitochondrial beta oxidation, long chain fatty acids (14 or more carbons) require a specific shuttle for import into the matrix. In the import machinery the fatty acid moiety of a fatty acyl-CoA is transiently bound to carnitine by carnitine acyltransferase I (CPTI) enzyme on the outer mitochondrial membrane. The conversion of fatty acids into carnitine esters commits the molecules for oxidative breakdown in mitochondria. Fatty acyl-carnitines enter the mitochondrial matrix by facilitated diffusion by acyl-carnitine/carnitine transporter of the inner mitochondrial membrane. On the inner mitochondrial membrane, the fatty acyl-carnitines are converted to intramitochondrial fatty acyl-CoA by CPTII. Depending on length of the acyl-chain, beta oxidation of the fatty acyl-CoAs yield a varying amount of two carbon acetyl-CoA units for TCA cycle and NADH and FADH<sub>2</sub> species for OXPHOS. (Kerner, 2000; Nelson and Cox, 2008, chapter 17).

### **2.1.2.2 Oxidative phosphorylation**

To synthesize ATP, the mitochondrial respiratory chain has to prime the reaction by generating a driving force, an electrochemical gradient across the mitochondrial inner membrane, the mitochondrial membrane potential (Mitchell, 1961). The membrane potential is mainly the net difference between concentration of protons (=hydrogen ions, H<sup>+</sup>) in the matrix (=low) and intermembrane space (=high). Protons are actively pumped in the intermembrane space by the respiratory chain enzyme complexes. The pumping is energized by transfer of electrons (e<sup>-</sup>) derived from the reduced cofactors NADH and FADH<sub>2</sub>. The electrons are shuttled throughout the respiratory chain, or sometimes called the electron transfer chain, to the final acceptor, molecular oxygen (=respiration). ATP synthase enzyme links the movement of protons down their electrochemical gradient (=protonmotive force) to covalent attachment of an inorganic phosphate to a molecule of ADP, generating ATP (Figure 2). (Saraste, 1999; Nelson and Cox, 2008, chapter 19).

The respiratory chain consists of multiprotein complexes (I-IV) and soluble electron carriers of inner membrane. Together, the respiratory chain



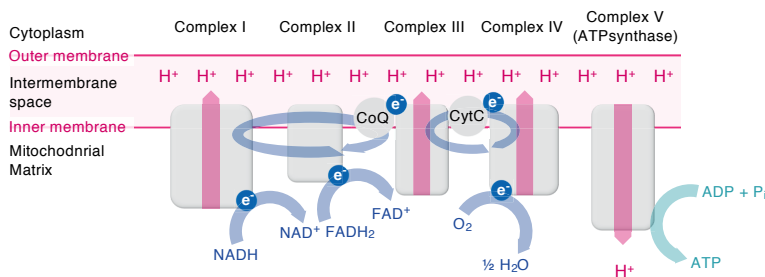
complexes and ATP synthase (Complex V) form the complete oxidative ATP production unit, OXPHOS.

Complex I and Complex II of the respiratory chain accept the electrons released from the breakdown of macronutrients and metabolites. Complex I, or NADH dehydrogenase, accepts electrons by oxidizing NADH and regenerating NAD<sup>+</sup>. Along the transmembrane part of the enzyme, electrons are transported through iron-sulfur clusters to the lipid-soluble electron carrier ubiquinone, or coenzyme Q<sub>10</sub>. In the process, four protons are pumped into the intermembrane space. Complex II, or succinate dehydrogenase, transfers electrons from FADH<sub>2</sub> to reduce ubiquinone, similarly as Complex I. Unlike other respiratory chain complexes, Complex II does not pump protons across the membrane and it also catalyzes oxidation of succinate to fumarate as part of the TCA cycle.

Complex III, or coenzyme Q : cytochrome c oxidoreductase, acts between two solute electron carriers of the respiratory chain trafficking in the inner mitochondrial membrane. Complex III accepts electrons from ubiquinol (CoQ), the reduced form of ubiquinone, and passes them on to cytochrome c (CytC). Complex IV, or cytochrome c oxidase, transfers electrons from cytochrome c to molecular oxygen. In the process, protons are pumped into the intermembrane space by both Complexes III and IV.

Complex V, or ATP synthase, catalyzes the enzymatic conversion of ADP to ATP. Complex V passes hydrogen ions through its transmembrane channel subunit. This movement of protons energizes conformational rotation in the catalytic unit. The alternating states of the catalytic unit either bind substrates ADP and inorganic phosphate (P<sub>i</sub>) or release a newly synthesized ATP molecule. To power the thermodynamically unfavorable enzymatic reactions of the cytoplasm, ATP needs to be transported across mitochondrial membranes by active antiporter function of ADP/ATP translocase.

(Saraste, 1999; Martínez-Reyes, 2016)



**Figure 2** Illustration of the mitochondrial OXPHOS complexes embedded in the inner mitochondrial membrane. Transfer of electrons (e<sup>-</sup>) from NADH and FADH<sub>2</sub> to molecular oxygen is coupled to pumping of protons (H<sup>+</sup>) from matrix to intermembrane space by Complex I, III and IV. Controlled passing of protons from intermembrane space back to the matrix energizes phosphorylation of ADP to ATP by ATP synthase.

Tight coupling of proton pumping by respiratory chain to ATP synthesis is not the only metabolic function of the proton gradient. Passing of protons through separate membrane channels of the inner membrane can uncouple the respiratory chain from Complex V. The rapid discharge of membrane potential generates heat. The dedicated uncoupling proteins of mammalian organisms (UCP1-3) have tissue-specific expression patterns and specialized functions in metabolic regulation, such as adaptive thermogenesis and ROS management (Hagen, 2002).

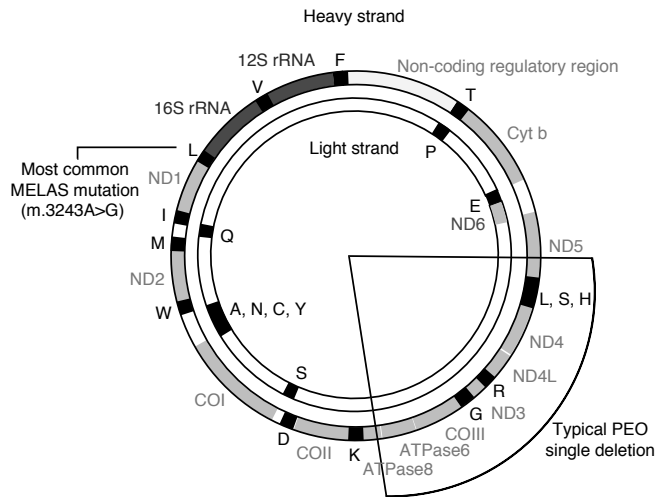
### **2.1.2.3 Reactive oxygen species**

Electron transfer reactions and reduction of oxygen by the respiratory chain has potential to generate highly reactive oxygen radicals, so called reactive oxygen species (ROS). Reactions between Complex I and ubiquinone and ubiquinol and Complex III involve a radical intermediate that has potential to transfer an electron to oxygen, generating a superoxide radical ( $O_2^{\cdot-}$ ) that can undergo further transformation to a hydroxyl free radical ( $\cdot OH$ ) (Zhao, 2019). In healthy cells, ROS production is readily controlled by cellular superoxide dismutase enzymes and glutathione peroxidase, converting excess superoxides to hydrogen peroxide and water. Impaired electron transfer due to respiratory chain complex mutations can critically increase the amount of ROS, therefore contributing to generalized cellular pathogenesis through oxidation of lipids, enzymes and nucleic acids and through complex alterations in cellular signaling cascades (Schieber, 2014). Therefore, extensive ROS and cellular damage has been suggested as pathophysiological hallmarks of primary mitochondrial diseases (Lenaz, 1998) but pathological adverse effects have been described also in other neurodegenerative disorders (Coyle, 1993). The various roles and importance of ROS, however, are not completely understood. ROS has shown to play important roles in sensing and signaling of normal physiological conditions and challenges (Chandel, 2007; Ahlqvist, 2015; Hämäläinen, 2015; Dogan, 2018), highlighting the necessity of a controlled ROS environment.

## **2.2 MTDNA AND MITOCHONDRIAL GENETICS**

The Mitochondrial genome, mtDNA, is organized as a supercoiled double-helical circular molecule. In humans and mice, mtDNA is approximately 16,600 base pairs in length. The two strands of mtDNA are called the heavy and light strands. Heavy strand is particularly GC-rich and mainly consists of coding regions, whereas the light strand is relatively gene-poor. Broadly, the mtDNA is extremely compact: gene areas generally lack introns and regulatory sequences are concentrated in a short non-coding regulatory region (or D-loop) containing promoter sequences for heavy and light strand transcription and origin of replication for the heavy strand.

MtDNA encodes 13 polypeptides of OXPHOS, 22 tRNA molecules and 12S and 16S rRNAs of mitochondrial translation machinery (Anderson, 1981; Falkenberg, 2018) (Figure 3). Only a minority of OXPHOS polypeptides and mitochondrial translation machinery, that altogether involve hundreds of proteins, are therefore encoded by the mitochondrial genome. The rest of the genes encoding mitochondrial proteins, including all subunits of Complex II, have translocated to the nuclear genome (Timmis, 2004), or were acquired during evolution to be part of OXPHOS complexes, mitoribosome, or their assembly. The reason for maintaining separate mtDNA genomes, requiring dual control of the mitochondrial energy metabolism, remains unclear.



**Figure 3** Genes encoded by human (and mouse) mtDNA. Examples of two common mtDNA disease mutations have been indicated. Abbreviations: Black genes with capital letters indicate the single-letter amino acid code of the corresponding tRNA, ND(x)=Complex I subunits, Cyt b=Complex III subunit, CO(x)=Complex IV subunits, ATPase(x)=Complex V subunits. Note that none of the Complex II subunits are encoded by the mtDNA.

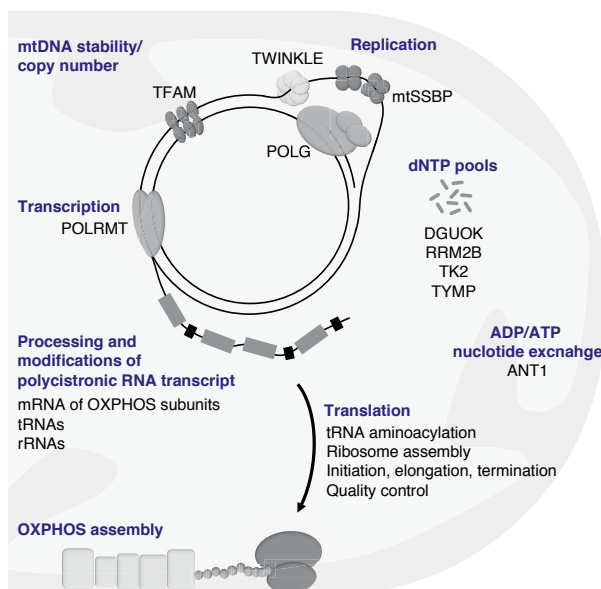
MtDNA is packed and organized into nucleoprotein complexes, called nucleoids. By super-resolution fluorescent microscopy, nucleoids have been shown to associate tightly with cristae formations of the inner mitochondrial membrane, and to consist mainly of a single mtDNA molecule and TFAM proteins (Brown, 2011; Kukat, 2011). TFAM (mitochondrial transcription factor A) is a mammalian mtDNA-associated protein that coats and packages mtDNA in a histone-like manner, and bends the promoter to allow transcription (Fisher, 1992). Experimental models have shown robust dependence of mtDNA stability and amount on TFAM expression levels (Larsson, 1998; Ekstrand, 2004). The role of the replication machinery and other proteins influencing mtDNA stability are discussed later in chapters 2.3 and 2.4.2.

Depending on the tissue type, each cell carries a large amount of mtDNA copies, from tens to several thousands of mtDNA molecules. If all mtDNA molecules are identical, the state is called homoplasmy. However, if e.g. mutated and healthy mtDNA molecules co-exist, the state is heteroplasmic. Many diseases have been reported to manifest only after a critical threshold of mutant mtDNA heteroplasmy level (Taylor, 2005). The mechanisms through which the cells can regulate the level of heteroplasmy or select between mtDNA molecules are not completely understood. In diseases, as well as in specific tissue environments and cell culture conditions, however, certain mitochondria and/or mtDNA haplotype have been shown to be selected for or against (Jenuth, 1997; Jokinen, 2013, 2016). For example, in stem cells, mitochondria have been reported to asymmetrically distribute between daughter cells to maintain stemness of the one receiving young mitochondria (Katajisto, 2015).

In reproduction, mtDNA follows uniparental transmission. In mammals, mitochondria of the sperm cells are actively degraded in the oocyte (Sato, 2013). Therefore, the mitochondria of the mother give rise to mitochondrial population of the progeny, with implications on the inheritance patterns of genetic mitochondrial diseases (Taylor, 2003) forensic sciences and anthropology. Evidence of paternally transmitted mtDNA in human pedigrees has been reported as a rare occurrence (Schwartz, 2004; Luo, 2018) but whether such mechanisms truly occur is still under debate in the mitochondrial genetics field, and the phenomenon is unlikely to have a major contribution in mitochondrial inheritance (McWilliams, 2019).

## **2.3 FUNCTIONAL OXPHOS AS RESULT OF SUCCESSFUL MITOCHONDRIAL PROTEIN SYNTHESIS**

Mitochondria have their own protein synthesis machinery devoted to synthesize the mtDNA encoded OXPHOS subunits. The synthesis of these 13 mtDNA encoded polypeptides is completely dependent on and regulated by nuclear encoded proteins that maintain and express mtDNA (Suomalainen, 2018). See Figure 4 for simplified representation of the steps and some key proteins involved in expression of the mtDNA.



**Figure 4** Overview of the mitochondrial protein synthesis and mtDNA maintenance, essential for functional OXPHOS assembly. Abbreviations: ANT1=mitochondrial ADP/ATP translocase 1, DGUOK=deoxyguanosine kinase, mtSSBP=mitochondrial single strand binding protein, POLG=polymerase gamma, POLRMT=mitochondrial RNA polymerase, RRM2B=ribonucleotide reductase regulatory TP53 inducible subunit M2B, TFAM=mitochondrial transcription factor A, TK2=thymidine kinase 2, TYMP=thymidine phosphorylase.

### 2.3.1 REPLICATION AND MAINTENANCE OF MTDNA

MtDNA can be replicated independently of cell cycle, even in post-mitotic cells (Clayton, 1982). Mitochondrial DNA replication is carried out by specialized enzymes, distinct from the nuclear DNA replication machinery. The minimal replisome of mtDNA (*in vitro*) consists of polymerase gamma (POLG,  $\alpha$  and  $\beta$  subunits), helicase TWINKLE and mitochondrial single strand binding proteins (mtSSBP) (Korhonen, 2004). The POLG has been thought to be the primary and only replicative polymerase and DNA repair enzyme in mitochondria (Bolden, 1977). However, increasing evidence during the last decade shows that alternative polymerases may also localize to mitochondria (Krasich, 2017). These polymerases have not been shown to have a major contribution in basal replication but could become essential during damage or stress related conditions. For example, PrimPol has been shown to assist re-priming of DNA replication after mtDNA damage (Stojković, 2016; Torregrosa-Muñumer, 2017). The TWINKLE helicase was originally found in a study that described a novel disease-causing mutation for PEO (see 2.4.2.2). The mammalian TWINKLE is homologous to the bacteriophage T7 gene 4 helicase/primase, but the mammalian enzyme is likely to only possess the DNA unwinding helicase function in the replicative fork (Spelbrink, 2001), while the mitochondrial RNA polymerase serves as the primase (Wanrooij, 2008). MtSSBP binds and protects single stranded mtDNA from nucleases and secondary structure formation, and functionally enhances POLG and TWINKLE processivity (Mignotte, 1985; Tiranti, 1995; Farr, 1999; Korhonen, 2003).

In the context of tissues *in vivo*, numerous additional to the minimal replisome proteins are known to regulate mtDNA maintenance. For example, mutations in OPA1 of mitochondrial fusion machinery cause mtDNA deletions (Amati-Bonneau, 2008; Hudson, 2008). Importantly, the replication and transcription of mtDNA are dependent on nucleotide availability. As mtDNA synthesis is continuous and occurs irrespective of the cell cycle in post-mitotic tissues, the salvage pathways of nucleotide synthesis in mitochondria are considered particularly important for mtDNA maintenance. Both cytoplasmic and mitochondrial dNTP pool regulation, and adenine nucleotide transportation, have been shown to be essential for mtDNA maintenance (Nishino, 1999; Kaukonen, 2000; Mandel, 2001; Saada, 2001; Fratter, 2011).

Alternative models of replication for the mtDNA have been described. The understanding is not complete and several modes may co-exist simultaneously (Holt, 2000, 2012). The first described and widely accepted strand-displacement model of mtDNA synthesis is asymmetric, originating from separate heavy and light strand replication origins (Robberson, 1972; Clayton, 1982). In the strand-displacement model, replication initiates first from the heavy strand origin of replication ( $O_H$ ), located in the non-coding regulatory region of mtDNA. Replication of the leading strand from the  $O_H$  proceeds approximately two thirds of the mtDNA length until it reaches the light strand origin of replication ( $O_L$ ). When the  $O_L$  is exposed, the lagging strand replication then proceeds to the opposite direction. RNA primers are needed for initiation of synthesis to start from  $O_H$  and  $O_L$ , synthesized by the mitochondrial RNA polymerase (POLRMT) (Xu, 1996; Wanrooij, 2008). In another mechanism, very similar to strand-displacement model, replication of the leading strand exposes the lagging strand for incorporation of RNA-intermediates that are subsequently replaced by, or converted to, DNA (Yasukawa, 2006). This RITOLS (RNA incorporated throughout the lagging strand) model implies that synthesis of both strands is asymmetric and uncoupled, but the main difference to the strand-displacement model is protection of not-replicated strand with RNA rather than mtSSBP. Furthermore, also a strand-coupled synthesis model has been described, where synthesis of both strands is simultaneous and symmetrical. The strand-coupled mode of replication was found alongside the other modes and likely utilized by the cell under certain conditions that require robust synthesis of new mtDNA molecules (Holt, 2000).

### 2.3.2 MITOCHONDRIAL GENE EXPRESSION

Central dogma of molecular biology implies transfer of information in sequential steps: DNA-encodes information to make mRNA (transcription), mRNA-code matches amino acids that form polypeptides (translation), further folded into functional proteins. The mitochondrial gene expression (transcription of mtDNA and mitochondrial translation) system shares

features of the bacterial origin, but is not completely analogous to either the prokaryotic or eukaryotic cytoplasmic processes.

Transcription of mtDNA originates from the non-coding region containing the heavy and light strand promoters (Chang, 1984). MtDNA is transcribed by the mitochondrial RNA polymerase (POLRMT) (Tiranti, 1997) and produces a long polycistronic mRNA, similar to prokaryotes. The polycistronic mRNA is processed by cleavage enzymes, allowing further modifications of mRNA and folding of tRNA molecules to secondary and tertiary structures (Hällberg, 2014).

During translation, the mRNA is read in three-base codons by the mitochondria. Each codon codes for an amino acid, START or STOP signal. The mitochondrial genetic code differs from the eukaryotic cytoplasmic code and is initiated exclusively with a formylated methionine, similar to bacterial translation (Hällberg, 2014). During the elongation phase of translation, each codon has a cognate mitochondrial encoded tRNA that brings the correct amino acid to the reaction. Mt-tRNAs, in turn, are aminoacylated by nuclear encoded mitochondrial aminoacyl-tRNA synthetases, mt-aaRS (Konovalova, 2013).

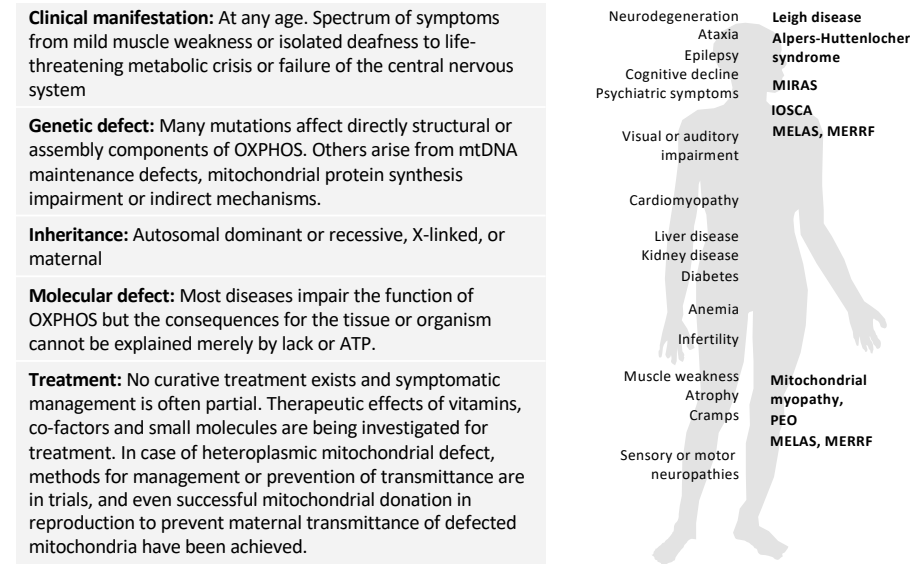
Synthesis of polypeptides by mitochondria takes place on the inner mitochondrial membrane. The mitochondrion has the traditional ribosome structure that consists of large and small subunits but has exceptionally high protein to rRNA ratio. In the translation process, numerous nuclear encoded initiation-, elongation- and termination factors are involved. The synthesis of the nascent OXPHOS polypeptides in mitochondria appears to be simultaneous to membrane insertion and folding, involving specialized assisting machineries and active quality control. (Hällberg, 2014; Suomalainen, 2018).

## **2.4 MITOCHONDRIAL DISEASES**

### **2.4.1 SPECTRUM OF CLINICAL AND GENETIC DEFECTS**

According to the current listings, 250-300 mitochondrial proteins are reported to carry disease-causing mutations. The most recent update of proteins located to mitochondria is around 1200 (Calvo, 2016), expanding the list of possible disease causing candidate genes. The most common mitochondrial diseases involve compromised function of OXPHOS. Even under the “OXPHOS umbrella”, however, mitochondrial diseases are clinically an exceptionally variable group of disorders. Therefore, individual disorders are rare, the rarest ones sometimes only described in a few pedigrees. If combined, however, estimates based on European populations state that prevalence of mitochondrial disease could be 1/4000, making them one of the most common inherited neurological or metabolic disorders (Gorman, 2015;

Saneto, 2017). The reason for the clinical diversity is poorly understood and mitochondrial diseases are currently mostly lack a cure. In summary, classical features of mitochondrial diseases (some of them are listed next to the symptoms in the illustration below) are summarized by the following statements (modified from Ylikallio, 2012; Ahmed, 2018):



The subsequent chapters introduce the clinical, genetic and molecular characteristics of the relevant mitochondrial diseases for this thesis. The diseases are divided into sub-groups according to the molecular nature of the genetic defect, all fundamentally compromising the function of OXPHOS. First, mutations that directly affect structure or assembly of the respiratory chain complexes are introduced. Second, mechanisms that compromise maintenance and expression of mtDNA are discussed.

### 2.4.2 PRIMARY OXPHOS DISORDERS

Clinically, respiratory chain mutations manifest early in life as severe neurological diseases. Biochemical assessment of tissue samples may reveal isolated complex deficiency. As mtDNA encodes only 13 polypeptides of the OXPHOS, nuclear mutations of the structural subunits of the catalytic cores and their assembly factors outnumber the mitochondrial mutations.

One example of a primary OXPHOS disease is Leigh or Leigh-like syndrome, also called ‘Leigh disease or subacute necrotizing encephalomyelopathy’ (OMIM entry #256000) (Leigh, 1951). Leigh syndrome is a classical mitochondrial disease, manifesting typically before two years of age with progressive symptoms, and having various genetic causes. Variable neurological symptoms in Leigh (for example psychomotor retardation,



hypotonia, ataxia, seizures) arise from specific bilateral lesions in the basal ganglia and thalamus, and associated abnormalities. Although the neurological symptoms dominate the clinical description of Leigh disease, skeletal muscle may show OXPHOS deficiency, and a biopsy sample can be used for diagnostic assays of mitochondrial function and mtDNA. Mutations in nuclear-encoded mitochondrial proteins for Complex I, II, III, or IV, mtDNA-encoded subunits of the ATP synthase and respiratory chain complex assembly factors are the main genetic causes of early onset Leigh disease. More rare causes involve defects of Coenzyme Q10 metabolism or the TCA cycle enzymes (Baertling, 2014; Rahman, 2017).

#### 2.4.1 MITOCHONDRIAL TRANSLATION DISORDERS

In biochemical assays, protein synthesis diseases usually manifest as generalized OXPHOS deficiency of single or multiple organs. Of the numerous mitochondrial protein synthesis factors, almost all are described with disease-causing mutations. However, defects in mitochondrial tRNAs and nuclear encoded mt-aARSs are the most prevalent causes of mitochondrial protein synthesis diseases (Suomalainen, 2018).

MELAS (OMIM entry #540000), or Mitochondrial myopathy, encephalomyopathy, lactic acidosis, and stroke-like episodes syndrome, is almost exclusively caused by m.3243A>G point mutation on mtDNA encoded *MT-TL1* gene (encoding for mitochondrial tRNA<sup>Leu</sup>) (Goto, 1990) (Figure 3). MELAS manifests as a complex multi-organ disease where most frequent clinical findings are strokes, epilepsy and exercise intolerance combined with finding of ragged red muscle fibers (excess proliferation mitochondria, a histological hallmark of mitochondrial disease) (El-Hattab, 2015). Surprisingly, mutations in different mitochondrial tRNA molecules do not cause similar diseases (Blakely, 2013). MERRF (OMIM entry #545000), or myoclonus epilepsy and ragged red fibers, is predominantly caused by m.8344A>G mutation in mitochondrial *MT-TK* gene of tRNA<sup>Lys</sup> (Shoffner, 1990), but is clinically distinct from MELAS with more restricted manifestation with seizures and myopathy, and shows a peculiar phenotype of symmetric lipomas in the neck-shoulder region. The different manifestations may arise from the different nature of defects in the protein synthesis machinery and the subsequent quality control needed for resolving the damage (Battersby, 2019), as well as tissue-specific demands and remodeling of metabolism. Furthermore, the prevalent m.3243A>G point mutation not only causes MELAS but also a spectrum of disorders and manifestations with variable severity, for example maternally inherited diabetes and deafness (MIDD) (van den Ouweland, 1994), PEO, mitochondrial myopathy, cardiomyopathy, migraine or cognitive impairment (Nesbitt, 2013). This spectrum of clinical manifestations for m.3243A>G has been shown to present poor correlation with heteroplasmy level, age or sex, although the heritability

of subset of symptoms are higher, suggesting strong modification by the nuclear background (Pickett, 2018).

In addition to mtDNA tRNA mutations, essentially all nuclear encoded mt-aaRS have been described with pathogenic mutations that are expected to impair mitochondrial protein synthesis (Konovalova, 2013; Sissler, 2017). The main clinical manifestation is often early-onset encephalopathy, but the spectrum of clinical manifestations ranges from cardiomyopathy to hearing loss, infertility, hematologic manifestations, and adult-onset myopathy (Konovalova, 2013; Suomalainen, 2018). The variable tissue specificity could be explained by unconventional non-translational activity of the mt-aaRS, already described for many of the cytoplasmic amino acid synthetases (Guo, 2013), but not yet for mammalian mt-aaRS (Tyynismaa, 2014). However, in yeast, overexpression of non-catalytic domain of mt-LeuRS was able to suppress growth retardation caused by the cognate mt-tRNA ablation, and similar results have been published for mammalian cells with the m.3243A>G mutation (Park, 2008; Francisci, 2011). Moreover, a human subject with pathogenic mt-tRNA<sup>Ile</sup> mutation remained clinically unaffected because of naturally higher expression level of mt-IleRS (Perli, 2012). Therefore, these evidence from both mt-tRNA and mt-aaRS diseases points to yet uncharacterized levels of regulation in mitochondrial translation and cellular amino acid metabolism, with implications on clinical manifestation.

#### **2.4.2 MTDNA MAINTENANCE DISORDERS**

Defects in mtDNA maintenance machinery can lead to pathological mutations, namely deletions or depletion of mtDNA. The causative genes for mtDNA depletion and deletion syndromes almost completely overlap but clinical presentations are often remarkably different, forming a clinical continuum with the manifestation depending on the age of onset of the disease.

In simple terms, mtDNA depletion syndromes are usually recessively inherited infantile-onset diseases that manifest with either hepatocerebral, neuro-gastrointestinal, encephalomyopathic or myopathic set of symptoms. Mutations in genes regulating the nucleotide pools and mtDNA replication compromise mtDNA maintenance and cause mtDNA depletion (El-Hattab, 2013). Yet, the most common single gene for mtDNA depletion syndromes is POLG, a leading cause of childhood-onset neurological disorders of mitochondrial origin, such as Alpers-Huttenlocher syndrome (Naviaux, 2004; Suomalainen, 2010; El-Hattab, 2013). Hepatocerebral manifestation due to mtDNA depletion is frequently caused by recessive mutations in deoxyguanosine kinase, or the replicative helicase TWINKLE, the latter manifesting as infantile-onset spinocerebellar ataxia syndrome, IOSCA (Nikali, 2005; Hakonen, 2008). Only recently, the third player of mtDNA minimal replisome, mtSSBP, was described with dominant and recessive mutations underlying multi-tissue mtDNA depletion in an optic atrophy disorder with kidney failure (Del Dotto, 2019).

MtDNA deletions can occur spontaneously, causing diseases without known involvement of mitochondrial or nuclear genes. The single deletions of mtDNA are typically sporadic in nature and occur early in the embryonic development (Marzuki, 1997). Most common single deletion associated with PEO (and Kearns-Sayre and Pearson syndromes) is 4977 base pairs in length, flanked by 13-base-pair direct repeats. The repeats have been proposed to predispose the replisome for slipping and mispairing the template, thereby generating the deletion. The mtDNA<sup>del4977</sup> removes four genes for subunits of Complex I, one gene for Complex IV, two genes for Complex V, and five genes for tRNAs (Shoffner, 1989) (Figure 3). In addition to sporadic deletions, mutations of the mtDNA maintenance factors (see Figure 4 and 2.3.1) cause a spectrum of adolescence or adult-onset ataxia syndromes or pure myopathies that are rather characterized by multiple mtDNA deletions (Spelbrink, 2001; Hakonen, 2005; Winterthun, 2005). The mechanisms of how mtDNA replication defects cause tissue-specific manifestations are not known, and may involve secondary genetic and environmental modifiers. Interestingly, POLG expression has been suggested to be regulated by a set of central nervous system specific complex enhancers and regulatory RNA species, offering new genomic insight for explaining the mechanisms of tissue specific manifestations (Nikkanen, 2018).

In this thesis, we have focused our studies on patients with AdPEO (OMIM entry #609286, PEOA3, AdPEO, PEO), caused by sporadic single mtDNA deletions or secondary multiple mtDNA deletions due to TWINKLE mutation. For AdPEO-like progressive multiple deletions and mitochondrial myopathy, a corresponding mouse model, Deletor, has been described in laboratory of A. Suomalainen-Wartiovaara (Tynismaa, 2005, 2010). The clinical and molecular pathology of the PEO patients and Deletor mouse is described in the following chapters.

### **2.4.3 MITOCHONDRIAL MYOPATHY CAUSED BY MTDNA DELETIONS: ADPEO AND DELETOR MOUSE**

Clinically, AdPEO patients manifest an adult-onset slowly progressive myopathy with extra-ocular muscle weakness and ptosis, combined with generalized exercise intolerance (Lewis, 2002) and sometimes psychiatric symptoms (Suomalainen, 1992). TWINKLE mutations were described as the underlying cause of the multiple mtDNA deletions in early 2000s (Spelbrink, 2001) but presence of the multiple deletions up to seven kilobases in size were already described earlier in muscle and brain of PEO patients (Holt, 1988; Yuzaki, 1989; Zeviani, 1989; Suomalainen, 1992, 1997). The deletions were frequently found to originate from the D-loop region with susceptibility to generation of fragments flanked by short repeats (Schon, 1989; Zeviani, 1989), similarly to single mtDNA deletion patients. Interestingly, these pedigrees showed autosomal inheritance of the diseases, indicating a nuclear gene causing mtDNA deletions, and later identified as being caused by mutations in

mtDNA maintenance proteins (Suomalainen, 1995; Nishino, 1999; Kaukonen, 2000; Spelbrink, 2001).

On molecular and biochemical level, the large-scale deletions of mtDNA compromise function of several subunits of OXPHOS complexes as well as mitochondrial tRNAs, manifesting as multicomplex respiratory chain dysfunction. The respiratory chain dysfunction in the affected muscle can be detected as cytochrome c oxidase (COX) negative fibers. The COX-negative fibers often show characteristic accumulation of mitochondria (modified Gomori trichrome staining of the nuclear encoded Complex II), called ragged red fibers (RRF or SDH positive fibers). (See Figure 17 for examples of COX-negative and SDH-positive muscle fibers). Ultrastructure of the mitochondria in the affected fibers is often compromised and electron microscopy reveals large, hypodense mitochondria with disorganized cristae and different inclusion bodies (Suomalainen, 1992; Tyynismaa, 2005; Vincent, 2016).

The Deletor mouse model of mitochondrial myopathy carries a corresponding mutation to certain AdPEO patients, a 39-bp duplication in murine Twinkle cDNA, causing an in-frame duplication of 13 amino acids (353–365) in Twinkle (=Twinkle<sup>dup353-365</sup>). The Twinkle<sup>dup</sup> transgene is expressed under a ubiquitous  $\beta$ -actin promoter, but the expression of mutant versus wild-type Twinkle is maximally 1:1, mimicking the situation in the autosomal dominant disease (Tyynismaa, 2005). The duplication locates in the linker domain of Twinkle. This mutation does not affect the Twinkle hexamer formation, but affects the helicase structure, causing occasional replicative fork stalling that may predispose mtDNA to deletion formation (Goffart, 2009).

The Deletor mouse was one of the first mouse models for mitochondrial diseases that accurately mimicked disease hallmarks seen in human patients. Deletor mouse findings resemble closely the findings in the PEO patients that carry the same mutation, on genetic, histological and biochemical levels. The mice manifest with progressive mtDNA instability and late-onset respiratory chain deficiency in the skeletal muscle, heart and distinct neuronal populations (Tyynismaa, 2005). Therefore, the Deletor has been useful in studies tackling the mechanisms of pathophysiology and stress responses, the main topics of all original publications in this thesis.

The biochemical and molecular pathogenesis of the Deletor mouse is well-characterized (Tyynismaa, 2010; publications of this thesis), and the model has proven to be useful in treatment studies (Ahola-Erkila, 2010; Yatsuga, 2012; Khan, 2014), out of which some have been subsequently applied in pilot clinical trials on patients (Ahola, 2016). The treatments have relied on dietary interventions and co-factor supplements designed to resolve the metabolic roadblocks caused by mitochondrial respiratory chain dysfunction in the muscle. For example, NAD-deficiency was found to occur as a consequence of mitochondrial myopathy in these mice, and supplementation with a precursor of NAD, B<sub>3</sub>-vitamin nicotinamide riboside (NR), was able to restore the shifted balance of NAD<sup>+</sup>/NADH. NR-treatment led to a marked increase in

mitochondrial biogenesis and rescued pathological findings in muscle of Deletor mice (Khan, 2014). This approach has also been applied in another mitochondrial disease model (Cerutti, 2014) and reported to alleviate general age-associated physical decline (Mills, 2016). Vitamin-B supplements are suggested to be safe and efficient in human subjects even in large doses (Conze, 2019) and several pilot clinical trials on those are ongoing in mitochondrial diseases ([www.clinicaltrials.gov](http://www.clinicaltrials.gov) and personal communication). Boosting of ketone body oxidation is another metabolic treatment that some mitochondrial disease patients have benefited from, especially in epileptic manifestation caused by OXPHOS deficiency (Kang, 2007). Pathological signs in muscle of Deletor were improved after consumption of ketogenic diet pre- and post-symptomatically (Ahola-Erkila, 2010). Mitochondrial myopathy patients responded to a low carbohydrate ‘modified Atkins diet’ with subacute muscle fiber damage specifically affecting the most sick muscle fibers and long-term effects showed slight improvement of muscle function (Ahola, 2016). These examples demonstrate the major effects of diet and vitamins as modifiers of metabolic roadblocks with potential to alleviate consequences of mitochondrial dysfunction. Furthermore, the examples underline the usefulness of pre-clinical models parallel to patient studies for rare diseases, although the inter-species differences in responses need to be considered.

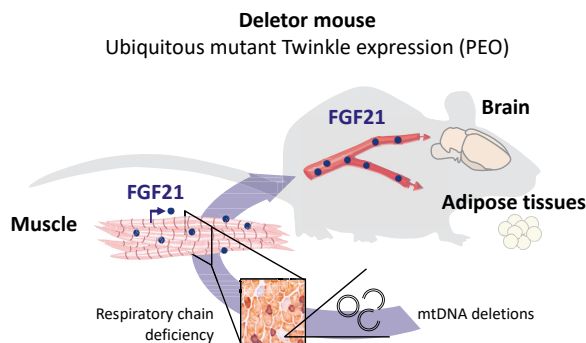
## **2.5 FGF21 – A CIRCULATING BIOMARKER WITH METABOLIC CONSEQUENCES**

### **2.5.1 STRESS SIGNALING AND CONCEPT OF SYSTEMIC ADAPTATION TO LOCAL MITOCHONDRIAL DYSFUNCTION**

In Deletor mouse, a chip-transcriptomic analysis of the affected muscle led to identification of a specific group of genes that carry a shared sequence in their promoter region, namely the amino acid response element (AARE) (Tyynismaa, 2010). The AARE-motif is known to be bound by the activating transcription factor (ATF) family of transcription factors responsive in stress conditions such as starvation (Stevenson, 2005; Nargund, 2012). Later on, this transcriptional response was demonstrated to be downstream of mTORC1 (mechanistic target of rapamycin complex 1), a major regulator of anabolic metabolism (Laplane, 2012; Khan, 2017). Interestingly, among the upregulated transcripts of Deletor muscle were two secreted factors, Fibroblast growth factor 21 (FGF21) and Growth and differentiation factor 15 (GDF15). Both proteins have thereafter been described as specific and sensitive serum biomarkers for mitochondrial diseases (Suomalainen, 2011; Yatsuga, 2015).

At the time when it was first linked to mitochondrial disease, FGF21 was reported as a metabolic regulator of systemic carbohydrate and fatty acid metabolism, described in detail in subsequent chapters. This indicated that

mitochondrial dysfunction in one tissue could mediate systemic adaptation of the whole organism (Figure 5). A similar concept of such ‘mitohormesis’ soon followed also in a *C. elegans* model of mitochondrial dysfunction (Durieux, 2011): upon inactivation of mitochondrial cytochrome c oxidase in neuronal cells of the worm, these cells initiated a protective local mitochondrial unfolded protein response, but the response was also found to occur in the gut of the worm where no primary defect occurred. Therefore, a hypothesis was put forward that a “mitokine” was secreted from the neurons, eliciting the mitochondrial unfolded protein responses (UPR<sup>mt</sup>) in distant tissues (Durieux, 2011; Nargund, 2012). Later on, the mitokine of the worm was reported to be a Wnt-ligand/EGL-20, expressed under activation of ATFS-1 transcription factor that promotes AARE-regulated transcription of mitochondrial heat shock proteins (Nargund, 2012; Zhang, 2018). The relevance of this worm response in mammals has so far not been clarified.



**Figure 5** The ‘Mitokine’ hypothesis presented for FGF21 in mammals, the central hypothesis of this thesis. FGF21 expression and secretion from skeletal muscle correlate with severity of respiratory chain deficiency in Deletor muscle. Systemic exposure to hormone FGF21 potentially regulates systemic adaptation and metabolism in response to the restricted tissue-specific disease manifestation.

### 2.5.2 FGF21 AND PHYSIOLOGICAL METABOLIC REGULATION

FGF21 belongs to the unconventional FGF19 subfamily of fibroblast growth factors that consists of FGF15/19 (mouse/human), FGF21 and FGF23 (Goetz, 2007; Beenken, 2012). The FGF19 subfamily lacks the characteristic heparin-binding domain typical for the FGF family, and regulates endocrine functions in metabolism, untypical for the canonical FGFs. FGF21 binds to a dimeric receptor consisting of generic fibroblast growth factor receptor type 1 (FGFR1) and  $\beta$ -Klotho, the latter defining the specificity and target tissues (Kharitonov, 2008; Suzuki, 2008; Yie, 2009; Yang, 2012) which are primarily the adipose tissues, liver, pancreas and hypothalamus (Ito, 2000; Ogawa, 2007).

In healthy physiology, FGF21 was originally reported as an acute starvation hormone, regulating the shift from fed to fasted state especially in mice

(Kharitononkov, 2005). In humans, a prolonged (seven days) fasting was reported to cause FGF21 secretion (Muisse, 2008). In fasting, FGF21 is expressed in response to peroxisome proliferator-activated receptor  $\alpha$  (PPAR $\alpha$ ) activation (Badman, 2007, 2009; Inagaki, 2007; Lundasen, 2007; Pothoff, 2009). PPARs are nuclear receptor transcription factors that get activated under conditions of energy deprivation, promoting catabolism of fatty acids and supporting ketogenesis (Kersten, 1999). Importantly, the PPAR $\alpha$ -mediated production of ketone bodies in the liver and systemic mobilization of fatty acids are shown to be highly dependent on FGF21 (Kharitononkov, 2005; Badman, 2007).

Studies on transgenic and pharmacogenetic models of FGF21 revealed that FGF21 is important in regulation of systemic metabolic homeostasis and body weight. Excessive FGF21 led to weight loss and resistance to diet-induced obesity, and lack of FGF21 predisposed mice to weight gain and metabolic syndrome (Kharitononkov, 2005, 2007; Coskun, 2008; Xu, 2009; Veniant, 2012). Pharmacological treatment with recombinant FGF21 protein, and synthetic variants, was shown to reduce body weight, improve insulin sensitivity, boost lipid metabolism and increase energy expenditure in rodents, non-human primates and humans (Coskun, 2008; Veniant, 2012; Gaich, 2013; Kharitononkov, 2013; Kim, 2015). Controversially, high levels of circulating FGF21 have been reported in humans with obesity, metabolic syndrome and fatty liver (Zhang, 2008; Chavez, 2009; Mraz, 2009; Mashili, 2011; Tynjismaa, 2011), suggesting decreased sensitivity to FGF21, similar to insulin-resistant conditions.

FGF21 was shown to freely pass through the blood-brain barrier in rodents (Hsueh, 2007; Tan, 2011) and injection of recombinant FGF21 to rat cerebrospinal fluid increased hepatic insulin sensitivity and metabolic rate (Sarruf, 2010), suggesting a regulatory role for FGF21 on metabolism through the central nervous system. Expression of the co-receptor  $\beta$ -Klotho was found to be concentrated in hypothalamus, and disturbance of FGF21- $\beta$ -Klotho interaction in the brain impaired the basic FGF21-mediated physiological functions (Bookout, 2013; Owen, 2013; Liang, 2014; Yilmaz, 2018; Hill, 2019). Since then, nervous system effects of FGF21 have been shown to be important in complex regulation of appetite for macronutrients and stimulants, such as sugar and alcohol (Schumann, 2016; von Holstein-Rathlou, 2016; Chen, 2017; Desai, 2017; Matsui, 2018; Søberg, 2018).

### **2.5.3 FGF21 AND NEUROPROTECTION**

Enhanced hepatic ketone production induced by FGF21 can be considered neuroprotective in nutrition-poor conditions. Additionally, periphery-derived FGF21 was reported to mediate hibernation or torpor-like state in rodents, a condition known to preserve and deviate energy for essential basic organ functions (Inagaki, 2007; Ishida, 2009). Further studies during the years have

revealed a spectrum of detailed functions for FGF21 in protection of the nervous system.

In transcriptional profiling of neuronal cultures exposed to common antidepressant and epilepsy drugs, lithium and valproic acid, intrinsic expression of FGF21 was induced (Leng, 2015). In that study, Leng et al. (2015), proposed that exogenously administered FGF21 prevented glutamate induced excitotoxicity and apoptosis in neuronal cultures through activation of Akt-1. Notably, FGF21 has been reported to support pancreatic beta-cell function via Akt-1 signaling cascades (Wente, 2006). In muscle cells, expression of FGF21 is reported to be downstream of Akt-1 (Izumiya, 2008). These studies highlighted that FGF21 can be expressed from a variety of tissues and act through conserved pathways that promote cell-survival and neuroprotection (Chiu, 2010).

Hyperglycemia-linked pathology in the nervous system has increased interest towards periphery driven modifiers of metabolism in the pathophysiology of neurodegenerative diseases. Very recently, FGF21 was shown to support maintenance of intact blood brain barrier in hyperglycemic of diabetic mice (Yu, 2019) and mediate neuroprotective actions through activation of cell-to-cell adhesion factor expression in traumatic brain injury (Chen, 2018). In another study on cerebral microvascular endothelial cells *in vitro*, FGF21 was shown to reduce apoptosis, alleviate inflammatory response and suppress damage by ROS and other hypoxia related pathological consequences (Wang, 2019). Protective effects of FGF21 have even been studied in a mouse model of Alzheimer's disease, where it was reported to ameliorate tau-aggregation and ROS pathology, thereby providing a mechanistic link to the regulation of hypoxia signaling (Chen, 2019). These examples highlight the accelerating efforts to unravel mechanisms of FGF21-mediated neuroprotection, with potential therapeutic opportunities for common neurodegenerative conditions that involve direct or secondary impairment of mitochondria.

## **2.6 METABOLOMICS IN RESEARCH AND DIAGNOSTICS OF MITOCHONDRIAL DISEASES**

Secondary metabolic perturbations, up-and downstream of the mitochondrial OXPHOS defects, are a poorly understood consequence of mitochondrial diseases. Several such changes are however used as biomarkers for disease. For example, elevated lactate or increased lactate/pyruvate ratio in blood or cerebrospinal fluid are markers of mitochondrial dysfunction (Haas, 2008), reflecting also cellular NADH/NAD<sup>+</sup> ratio (Nelson and Cox, 2008, chapter 14). However, these markers are not raised in all mitochondrial disease. Furthermore, for example levels of lactate and pyruvate are also elevated in response to other physiological and pathophysiological stimuli such as exercise, metabolic crisis and inflammation. Profiling of blood total amino



acids or carnitine and acyl-carnitines have been utilized to indicate mitochondrial disease. Acyl-carnitine profiling permits identification of secondary fatty acid oxidation defects that may occur in primary OXPHOS disorders (Haas, 2008) but are also markers of primary fatty acid oxidation diseases (Sim, 2002). Several markers of cellular metabolic disturbances have therefore been utilized routinely but with limited diagnostic sensitivity and or specificity.

### **2.6.1 MASS SPECTROMETRIC PROFILING**

Mass spectrometry (MS) is an analytical technique that measures the mass-to-charge ratio of ionized molecules. Basic parts of a MS are ionizer, mass analyzer and detector. Results are presented as a mass spectrum, from which the identity of target molecules or isotopic signature of a sample can be determined. Several pre-separation techniques of the targets can be combined with mass spectrometry. Popular method in biological research is liquid chromatography (LC) or high-performance liquid chromatography (HPLC) in combination with MS, LC-MS. Liquid chromatography separates complex biological samples according to favored chemical or physical properties, increasing the sensitivity of the subsequent MS identification (Gohlke, 1959). Modern targeted MS methods utilize multiple stages of tandem mass spectrometry for ions of specific mass, defined by standard molecules derived from previous analyses. This enables (semi) quantitative detection of  $1 \times 10^2$  magnitude of low molecular weight metabolites in a single run, providing possibilities for diagnostic use (Nandania, 2018).

Easily accessible body fluids, serum, plasma and urine, have been used to recognize signature patterns of single or multiple metabolite characteristics for certain mitochondrial diseases (Buzkova, 2018; Esterhuizen, 2019). Metabolic analysis of patient tissues can direct the diagnostic process, but better knowledge of the metabolic rearrangements is still required. However, knowledge of metabolism has broadened the view on pathophysiological mechanisms of mitochondrial diseases and might offer possible therapy targets in the future.

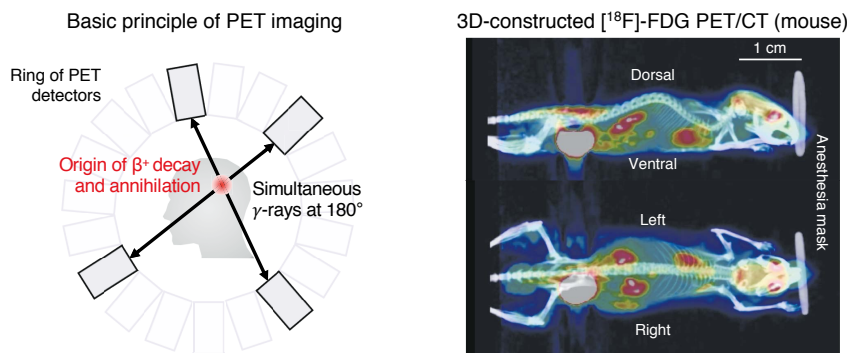
### **2.6.2 STABLE ISOTOPE LABELING WITH [ $^{13}\text{C}$ ]**

Metabolic flux analysis enables tracking the fate of isotope-labeled substrate carbons, reflecting dynamics of metabolism, and giving insight into catabolic and anabolic reactions *in vivo* or in cell cultures. For example,  $^{13}\text{C}$  is a naturally occurring heavy, non-radioactive, rare isotope of carbon,  $^{12}\text{C}$  being the most abundant one in nature. Detection of different isotopic atoms in a molecule can be done with nuclear magnetic resonance (NMR) or mass spectrometry-based analyses. Further analysis is based on advanced mathematical solutions and advanced computing to understand the metabolic networks. (Wiechert, 2001; Dai, 2017).

A common metabolic flux experiment, tracking nutrient usage, utilizes labeled glucose. Glucose can be labeled uniformly for all six carbons, [U-<sup>13</sup>C]-glucose, or on definite positions chosen for specific cellular pathways (such as glycolysis or the pentose phosphate pathway). The model system, cell culture or an animal, is provided with labeled substrate and the fate of labeled element is tracked among target molecules. In mass spectrometry, the labeled substrates are referred as mass isotopomers, (m+X), X being the number of substituted elements in the structure. For example, [U-<sup>13</sup>C]-glucose can yield to wide variety of (m+X) products starting from glycolysis, TCA-cycle and all subsequent and branching biosynthetic processes. Rapid metabolic processing and numerous fates of universal nutrient molecules make metabolic flux analysis technically challenging. Especially the live-cell labeling steps are prone to variation through experimental design, handling and biological variation, highlighting the need for proper experimental planning and use of appropriate controls. (Dai, 2017).

### **2.6.3 POSITRON EMISSION TOMOGRAPHY**

Positron emission tomography (PET) is an important quantitative non-invasive imaging method in medicine. Positron beta emission ( $\beta^+$ ) derives from decay of radionuclides, in which the number of protons exceed the number of neutrons. In  $\beta^+$  emission, two positrons are emitted to opposite directions, close to 180° angle. In the context of biological systems, such as the human body, the positrons annihilate with electrons, releasing two gamma particles in the same direction of movement as the original positrons. PET scanners detect the gamma-rays ( $\gamma$ ) that derive from indirect positron emission. Two gamma particles arriving to the PET detectors simultaneously in 180° angle are recorded. Information from multiple ring-organized is integrated to construct a 3D image of annihilation events. In most medical PET-scanners, a parallel X-ray based cross sectional computed tomography (CT) is overlaid with the PET image for a better spatial reconstruction (Dahlbom, 2017) (Figure 6).

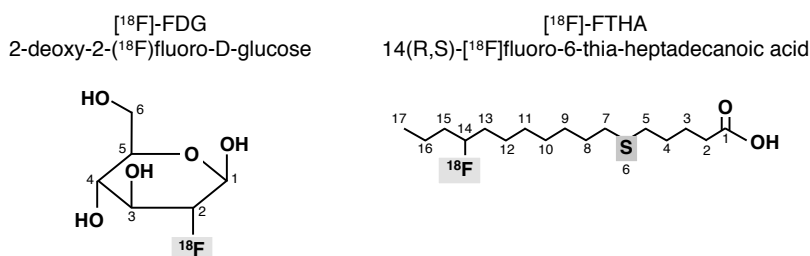


**Figure 6** **Left:** Schematic illustration of the basic principle of PET imaging. **Right:** Example of a reconstructed PET-image of [ $^{18}\text{F}$ ]-FDG tracer overlaid with a CT scan. Rainbow color indicates [ $^{18}\text{F}$ ]-intensity in soft tissues, white skeleton is reconstructed from the CT-scan.

Resolution of PET scanners is limited by the distance the positron travels before annihilation event, usually 0.5-8 millimeters (Saha, 2010; Granov, 2013). This can be a challenge for preclinical studies of small laboratory animals, such as mice. In small tissues or body fluid samples, accumulated radioactivity during the uptake period can be measured by gamma counters where gamma rays from beta decay in tissue interact with a crystal (*ex vivo*). As a result, light is emitted and converted to an electrical signal. The gamma counts are corrected for radionuclide decay (half-life and delay) and reported as a percentage of the injected dose per gram of tissue (%ID/g) or as an injected dose corrected for body weight and radioactivity per gram of tissue (SUV, standard uptake value). For samples that need resolution in the scale of sub-organ level, an autoradiograph of accumulated emission can be detected from planar surfaces. In autoradiography, beta/gamma emission from tissue slices is exposed on X-ray film in tight contact with the specimen, and the films are scanned for image reconstruction (Zanzonico, 2012). Such applications involve different organ-tumor studies, localized uptake of labeled metabolites or receptor binding in rodent brain, for example. After the exposure, the samples are suitable for subsequent histological analysis (see Figure 15 for an example of an autoradiograph of mouse brain).

In PET and *ex vivo* applications, ultra-short-lived radionuclides are used with half-lives ranging from a couple of minutes to hours. Short-lived nuclides are produced by radiochemical laboratories with cyclotrons that bombard the target substance with an accelerated, charged particle beam. Radio-isotopes of carbon ( $^{11}\text{C}$ ), nitrogen ( $^{13}\text{N}$ ), oxygen ( $^{15}\text{O}$ ) and fluoride ( $^{18}\text{F}$ ) are frequently used beta emitters with a short half-life. The radionuclides are chemically linked to biological or drug-based ligands responsible for targeting of the emitters. In *in vivo* studies, each radiotracer also has a biological half-life, based on the metabolic properties of the carrier/ligand in the body (such as fecal and urinary excretion or perspiration) (Saha, 2010; Granov, 2013).

In tumor imaging and metabolic activity recordings, glucose analog [ $^{18}\text{F}$ ]-FDG is the most widely used tracer. The [ $^{18}\text{F}$ ]-FDG, or [2-deoxy-2-( $^{18}\text{F}$ )fluoro-D-glucose], has radionuclide  $^{18}\text{F}$  substitution of a hydroxyl group (OH) at the C2 carbon of the glucose molecule (Figure 7). Substitution of the OH-group with  $^{18}\text{F}$  makes the labeled glucose molecule detectable in PET, and prevents further metabolism after the first irreversible phosphorylation step of glycolysis by hexokinase/glucokinase. [ $^{18}\text{F}$ ]-FTHA, or [14-(R,S)- $^{18}\text{F}$ fluoro-6-thia-heptadecanoic acid], is a mimetic of long chain fatty acids (Figure 7).  $^{18}\text{F}$  at carbon C14 enables detection and sulfur substitution on the carbon C6 causes metabolic trapping of FTHA after initial commitment to the beta-oxidation pathway but FTHA can undergo intracellular and extracellular reactions of fatty acid pools (Takala, 2002; Ci, 2006; Guiducci, 2007; Croteau, 2016).



**Figure 7** Chemical structures and modifications of glucose and palmitic acid analogs, [ $^{18}\text{F}$ ]-FDG and [ $^{18}\text{F}$ ]-FTHA radiotracers, respectively. Numbers indicate atoms of the carbon or carbon/sulfur backbone.

### 3 AIMS OF THE STUDY

The general aim of this thesis was to characterize the metabolic alterations and pathophysiology of mitochondrial dysfunction with special emphasis on the effects of the metabolic hormone FGF21. Specific aims were as follows:

1. To characterize in detail the metabolic remodeling of the affected muscles in mitochondrial disease caused by mtDNA instability
2. To clarify the local and systemic effects of chronic exposure to the hormone FGF21 on the pathophysiology
3. To examine the diagnostic use and mechanism of induction for FGF21 (and GDF15) in different mitochondrial diseases

## 4 MATERIALS AND METHODS

All method details are presented in the original publications and briefly described here.

### 4.1 ETHICAL STATEMENTS AND LICENSES

The Ethical Review Board of Helsinki University Central Hospital approved the sample collection and analysis of patients and controls, who gave their written informed consent, according to the Declaration of Helsinki. Human subjects were recruited to studies and sampled in the Helsinki University Central Hospital, and part of the samples in study III were obtained from international collaboration centers. The National Animal Review Board and regional State Administrative Agency for Southern Finland approved the animal studies of this thesis and experiments were performed following the European Union Directive.

### 4.2 MOUSE MODELS

Deletor mice with a comprehensive FGF21 knockout were generated for this study by sequential crosses with existing transgenic mouse lines. Heterozygous *Twinkle*<sup>dup 353-365</sup> (Deletor) males and PGK-Cre females (Lallemand, 1998) were crossed with *Fgf21*<sup>LoxP/LoxP</sup> animals (Potthoff, 2009). The (Deletor x *Fgf21*<sup>LoxP/LoxP</sup>) males were then crossed with (PGK-Cre x *Fgf21*<sup>LoxP/LoxP</sup>) females. All genotypes were born in the expected Mendelian ratios and were viable until euthanasia at 22-24 months of age. The population was maintained with crosses between Deletor-FKO males and FKO females. Additionally, six previously characterized transgenic and knockout mouse models were obtained through collaboration (phenotype of the mice is described in section 5.7). Sample collection, preservation and shipping conditions were harmonized and analyses were performed in Biomedicum Helsinki according to materials and methods.

### 4.3 TISSUE COLLECTION

Mice were euthanized with gradual inhalation of excess carbon dioxide. Post-mortem blood collection through heart puncture was performed and tissues collected within 5-15 minutes of death of the mouse. The collected tissues were snap frozen in liquid nitrogen and stored at -80°C, or processed for histological analyses as described in methods.

#### **4.4 IN VIVO UPTAKE ASSAYS OF <sup>18</sup>F-LABELED GLUCOSE AND FATTY ACID ANALOGS**

Prior to all tracer uptake assays, food was removed from cages three hours before. For fasting experiments, food was removed at 7-9 pm and the uptake assay performed the next morning, after 24 hours of fasting. For exogenous FGF21 treatment experiment, animals were injected (intraperitoneally) with 0.06 mg/kg of recombinant mouse FGF21 or saline four hours prior to the tracer assay. (See production of r-FGF21 in original publication I.)

Assays were performed in isoflurane inhalation anesthesia and body temperature was controlled with heating pads. The tracers [<sup>18</sup>F]-FDG or [<sup>18</sup>F]-FTHA were injected to the tail vein in total activity of 5 MBq. PET/CT scans were performed with Inveon multimodality PET/CT machinery (Siemens). Standard uptake values (SUV) (Deleye, 2016) were calculated from reconstructed PET images (Inveon Research Workplace visualization program, Siemens), or by using a manual gamma counter measurement of emitted gamma rays from excised tissues. Quantitative phosphor imaging autoradiography of brain was performed on coronal brain cryosections (20 µm) exposed on an imaging plate (Fuji BAS-TR2025) for approximately 3.5 hours. The plates were scanned with a Fuji BAS-5000 Phosphor imager and signal densities (photostimulated luminescence per unit area, PSL/mm<sup>2</sup>) obtained with an AIDA image analyzer.

#### **4.5 IN VIVO [U-<sup>13</sup>C] GLUCOSE FLUX**

For *in vivo* tracking of glucose 0.7 mg/g dose of [U-<sup>13</sup>C]-glucose (Cambridge Isotope laboratories, CLM-1396) was administered to the mice via tail vein. The mice were sacrificed and tissues collected after 5, 10 and 15 minutes of uptake. The samples were frozen and the metabolome analyzed by targeted selected reaction monitoring liquid chromatography-tandem mass spectrometry (LC-MS/MS) on Agilent 6460 QQQ.

#### **4.6 CELL CULTURE**

All cells were maintained in a humidified chamber at 37°C in 5% CO<sub>2</sub>. Culture conditions involved nutrient rich Dulbecco's Modified Eagle's Medium DMEM (Sigma-Aldrich), supplemented with fetal bovine serum, pyruvate, penicillin/streptomycin and uridine, in presence or absence of actinonin for mitochondrial translation inhibition (up to 24 hours). Gene silencing in fibroblasts was performed using the Polyplus jetPRIME transfection kit and SilencerSelect (ThermoFisher) siRNAs against scramble control, ATF3, ATF34 and ATF5.

## 4.7 QUANTITATIVE REAL-TIME PCR

RNA was extracted by standard Trizol-chloroform precipitation. Briefly, frozen tissue (20 mg in 1 ml of Trizol reagent) was homogenized with the Fast-Prep w-24 Lysing Matrix D (MP Biomedical) and Precellys w-24 (Bertin Technologies). One microgram of RNA was DNase digested and reverse transcribed (Maxima first strand synthesis kit). Quantitative PCR amplification was performed with the IQ SybrGreen kit (Bio-Rad) on the CFX96 Touch qPCR system (Bio-Rad). The relative expressions of genes of interest were normalized to the level of  $\beta$ -actin expression. All primer sequences can be found from the original publications.

## 4.8 RNA SEQUENCING

RNA from the patient muscle samples were extracted as in 4.7 but further purified with the RNA purification kit (RNeasy; Qiagen). A total of one microgram of total RNA was used for the global transcriptomics analysis performed by the Beijing Genomic Institute (BGI). 50 base pair paired-end sequencing on Illumina sequencing platform was performed with approximate coverage of 26 million reads per sample. The reads were aligned against the GRCh38 Human genome assembly using STAR 2.5.0a. Read count was obtained with htseq-count, HTSeq 0.10.0, in union overlap resolution mode. Differential gene expression analysis was done with the Bioconductor DESeq2 1.18.1 package. The fold changes in binary logarithmic scale and the respective p-values were extracted for pairwise comparisons.

## 4.9 MITOCHONDRIAL DNA ANALYSES

Total DNA was extracted using Phenol-chloroform-ethanol precipitation. Quantitative PCR (see 4.7) of DNA was used to analyze the copy number of mtDNA in relation to the genomic DNA. MtDNA deletion load was estimated with one-well triplex qPCR with 5'probed mtDNA primers ROX for D-loop, HEX for ND1 and FAM for ND4) (Rygiel, 2015). Visualization of the deleted mtDNA fractions was done with a long-PCR amplification protocol for mtDNA. All primer sequences can be found from the original publications.

## 4.10 WESTERN BLOT

Frozen tissues (10-20 mg) were homogenized in 50 mM Tris, 150 mM NaCl buffer with pH 7.6 with the Fast-Prep w-24 Lysing Matrix D (MP Biomedical) and Precellys w-24 (Bertin Technologies). Proteins were extracted with 1% Triton-X after homogenization. Proteins were run on SDS-PAGE and blotted on PVDF membranes (Trans-Blot Turbo transfer system, BioRad). Proteins



were visualized with antibodies listed in detail in the original publications after overnight incubation at +4°C.

## **4.11 HISTOLOGY**

Immunohistochemistry, histochemistry, and immunofluorescence stainings were performed with formalin or paraformaldehyde fixed, paraffin embedded tissues. For immunological staining, antigen retrieval with heating in citric acid was performed and antibody staining performed according to standard protocols following instructions by the manufacturer. Details and list of antibodies used can be found in the original publications.

Histochemical enzyme activity (COX/SDH) analysis and Oil-red-O staining for fat content were made from fresh frozen samples, embedded in Tissue-Tek O.C.T. mount, frozen in isopentane bath under in liquid nitrogen cooling. The staining procedures were performed according to (Ahola-Erkkila, 2010) and described in detail in the original publications.

## **4.12 METABOLOMICS**

Targeted metabolomic analyses were performed from 20 mg of tissue material using Waters Acquity ultra performance liquid chromatography (UPLC) and triple-quadrupole mass spectrometry according to (Nandania, 2018).

## **4.13 SERUM BIOMARKER MEASUREMENTS**

Measurement of serum biomarkers was performed with enzyme linked immunosorbent assays (ELISA) according to the manufacturer's instructions. Standard human serum or plasma were stored in -80°C prior to analysis. The primary kit for patient samples was FGF21 ELISA from BioVendor, and the results were validated with FGF21 kit from R&D Systems. The GDF15 ELISA kit was provided by R&D Systems. Mouse serum was obtained from terminal heart puncture sample. The whole blood was let to clot for 15 minutes at room temperature, and then centrifuged for 3000x g for 15 minutes at +4°C. Quantikine Mouse Fgf21 immunoassay from R&D Systems was used for the measurements. Absorbance measurements were made with the SpectraMax 190 absorbance microtiter plate reader.

## **4.14 STATISTICAL ANALYSES**

Statistical analyses and graphical representation were performed with the GraphPad Prism v.6.0 or v.7.0 software.

Student's t-test or ANOVA were used to compare experimental groups. In the biomarker study with patients, the normal cut-off values for serum concentrations were determined as the 95-percentile of healthy controls. Kruskal-Wallis test was used for multiple comparisons between the study groups.

Outlier analysis was done using GraphPad's ROUT method (Q=1%). Multiple testing correction for the metabolomics analyses was performed via the Benjamini-Hochberg method, using a corrected p-value below 0.2 as the threshold for significance.

## 5 RESULTS AND DISCUSSION

### 5.1 MTDNA MAINTENANCE DEFECTS IN THE SKELETAL MUSCLE REMODEL ONE CARBON METABOLISM (III\*)

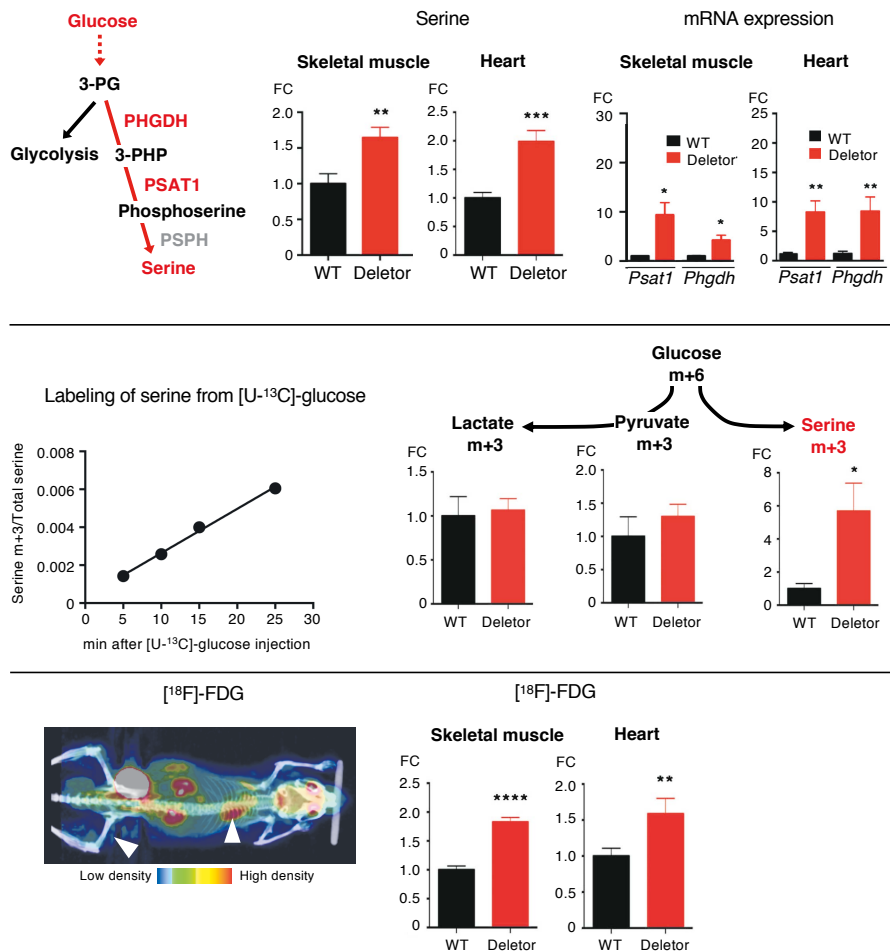
The genetic cause for mitochondrial myopathy of the patients studied in this thesis (publications I and III\*) was either 1) a single primary sporadic mtDNA deletion or 2) mutations in the nuclear-encoded TWNK, leading to multiple mtDNA deletions. The latter is analogous to the Deletor mouse model. Both primary and secondary mtDNA deletions are associated with mitochondrial dysfunction and respiratory chain deficiency in the affected muscle tissues (see 2.4.2 and 2.4.3 for details).

#### 5.1.1 INCREASED GLUCOSE UPTAKE IN DELETOR MUSCLE SUPPORTS SERINE DE NOVO SYNTHESIS

To study the metabolic status of the affected muscle tissues, we performed targeted quantitative mass spectrometry-based analysis of ~100 metabolites in PEO patient and Deletor muscle samples. The human and mouse metabolomes showed a significant increase in the overall levels of amino acids, as reported before for the Deletor mouse (Ahola-Erkkila, 2010). In particular, levels of serine and glycine were highly induced in the muscle and heart of the Deletors. Expression of the serine synthesis enzymes phosphoglycerate dehydrogenase and phosphoserine phosphatase (*Phgdh* and *Psat1*) were reported to be induced in the Deletor muscle previously, when a robust induction of genes regulated by amino acid response element (AARE) was described in the mouse skeletal muscle (Tynnismaa, 2010) (2.5.1). Here we show that in the skeletal muscle and heart of Deletor mouse a high level of serine is accompanied with induced expression of the serine biosynthetic enzymes (Figure 8).

PHGDH and PSAT1 divert 3-phosphoglyceric acid (3-PG), a three-carbon intermediate of glycolysis, towards *de novo* synthesis of serine. With a [U-<sup>13</sup>C]-glucose (glucose m+6) flux assay we were able to show linear labeling of serine (serine m+3) during 5-25 minutes of uptake in the heart of the Deletor. Remarkably, incorporation of glucose carbons to serine in Deletor heart was significantly increased compared to control mice without changes in the labeled glycolysis end-products, pyruvate and lactate (Figure 8).

To see whether increased serine biosynthesis influenced the total demand of glucose in the muscles, we did positron emission tomography (PET) using radiolabeled glucose analog, [<sup>18</sup>F]-FDG. In the [<sup>18</sup>F]-FDG PET, we detected a significant increase in glucose uptake to the Deletor muscle and heart over the 25 minutes of recording (Figure 8).



**Figure 8** **Upper panel:** Schematic presentation of glucose usage for serine biogenesis, red indicates induction in Deletor muscles (PSPH, phosphoserine phosphatase, was not measured). Steady state serine levels and mRNA expression of serine *de novo* synthesis enzymes PHGDH and PSAT1 in muscle tissues. **Middle panel:** Linear labeling of (m+3) serine from [U-<sup>13</sup>C]-glucose during the 25-minute uptake period in Deletor heart. Quantification of (m+3) lactate, (m+3) pyruvate and (m+3) serine in Deletor heart. **Lower panel:** [<sup>18</sup>F]-FDG biodistribution in a mouse, arrows point to skeletal muscle and heart, quantified on right. Statistical significance: \*= $p<0.05$ , \*\*= $p<0.01$ , \*\*\*= $p<0.001$ , \*\*\*\*= $p<0.0001$ . Abbreviations: 3-PG=3-Phosphoglyceric acid, 3-PHP=3-phosphohydroxypyruvate.

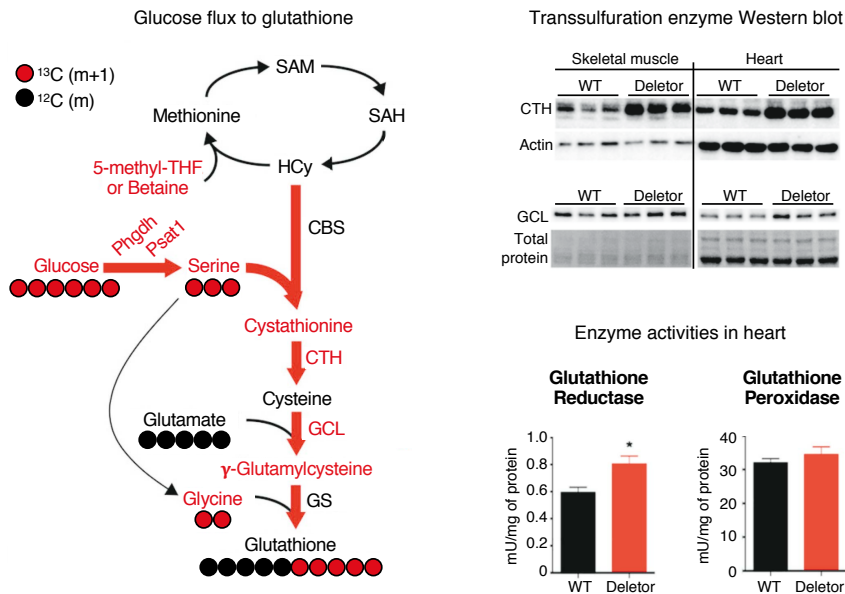
In summary, these results of the steady state and flux metabolomics together with [<sup>18</sup>F]-FDG PET suggest that the excess glucose in mitochondrial myopathy muscle was not directed to glycolysis for compensation of mitochondrial respiratory chain deficiency but was used to synthesize serine. This finding revealed a previously uncharacterized metabolic adaptation of the post mitotic muscle in mitochondrial disease.

### **5.1.2 SERINE IS SHUTTLED TO DRIVE TRANSSULFURATION IN DELETOR MUSCLE**

Analysis of evolutionary conservation can reveal pathways that show functional interdependence with each other. As PHGDH has been reported to be the determinant of 3-PG fate towards serine also in cancer cells (Locasale, 2011) (Figure 8), we utilized a co-evolution analysis tool CLIME (clustering by inferred models of evolution, <http://gene-clime.org>) (Li, 2014) to predict pathways dependent on PHGDH driven serine synthesis. The analysis strongly suggested co-evolution with the transsulfuration enzymes cystathionine gamma lyase (CTH) and cystathionine beta synthase (CBS). In a western blot, CBS was not detectable in muscle and heart tissues but CTH and another transsulfuration enzyme, glutamate-cysteine ligase (GCL), showed increased expression (Figure 9).

In transsulfuration, cysteine and glutathione are produced from homocysteine, serine, glutamate and glycine. In the [U-<sup>13</sup>C]-glucose flux assay of Deletor heart, we detected efficient and rapid labeling of (m+5) glutathione, indicating incorporation of glucose carbons through serine and glycine (Figure 9). In the steady-state targeted metabolomic analysis, transsulfuration intermediates cystathionine and gamma-glutamylcysteine were increased, supporting the drive for glutathione synthesis (Figure 9).

Glutathione exists in a dynamic balance of oxidized and reduced forms in a cell. Major consumers of reduced glutathione are the ROS-scavenging enzyme glutathione peroxidase and glutathione reductase which supports reductive biosynthetic reactions. In Deletor heart we detected increased glutathione reductase and normal glutathione peroxidase activities (Figure 9), implying that the increased production of glutathione is utilized in synthetic reactions rather than counteracting oxidative stress, often linked to mitochondrial respiratory chain deficiency (Kirkinezos, 2001; Atkuri, 2009).



**Figure 9** **Left:** Glutathione synthesis and reactions in Deletor muscle and heart. Flux of glucose carbons (red circles) throughout glutathione synthesis pathway in Deletor muscle and heart. Red text indicates increased metabolites in the steady state targeted metabolomics, Western blot or qPCR. **Right, top:** Western blot of transsulfuration enzymes in Deletor skeletal muscle and heart. **Right, bottom:** Enzyme activities of major enzymes that utilize reduced glutathione, glutathione reductase and glutathione peroxidase. Statistical significance: \*= $p < 0.05$ .

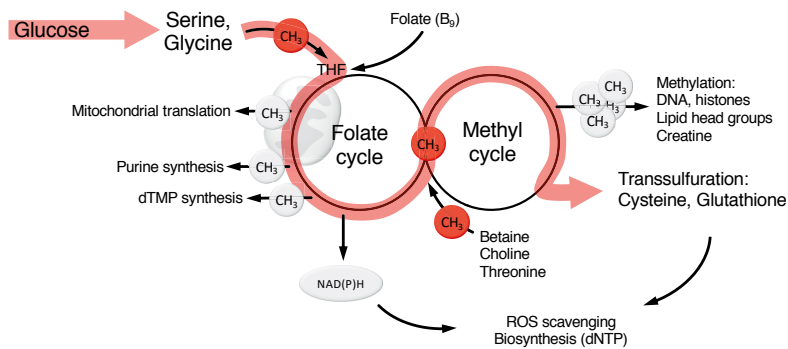
*De novo* deoxy nucleotide (dNTP) synthesis by ribonucleotide reductase is one of the many reactions dependent on reduced glutathione availability (Sengupta, 2014). In the muscle of Deletor, we indeed found increased and imbalanced dNTP pools, possibly reflecting the serine driven boosting of glutathione synthesis. A similar response was also reported in a mammalian cell culture model with acute mtDNA depletion (Bao, 2016). Intriguingly, serine biosynthesis has been shown to be essential for growth and survival of multiple cancer cell lines, ultimately through support of active nucleotide production for rapid proliferation (Locasale, 2011; Possemato, 2011; Locasale, 2013; Labuschagne, 2014; Mehrmohamadi, 2014; Pacold, 2016). Together, our findings suggest induction of nucleotide metabolism in a post-mitotic tissue with mtDNA replication defects, mimicking the metabolic adaptations of proliferative cancer cells.

In post-mitotic tissues, the main user of dNTPs is the mtDNA and impaired nucleotide pool regulation is a major cause of mitochondrial deletion and depletion syndromes (Suomalainen, 2018). Moreover, excess nucleotides in an already imbalanced nucleotide pool environment have been shown to be genotoxic in mice deficient of pyrimidine nucleotide conversion (Garcia-Diaz,

2014). In Deletor, we did not detect increased point mutation load, at least in the mtDNA. Therefore, it remains to be studied whether boosting of dNTP pools plays a protective or disruptive role in pathophysiology of primary mtDNA deletion diseases.

### 5.1.3 METABOLIC FINGERPRINT OF MITOCHONDRIAL MYOPATHY SUGGESTS ALTERED ONE CARBON METABOLISM

One-carbon metabolism refers to reactions that transfer single carbon units (1C) in the form of methyl groups ( $\text{CH}_3$ ) for biosynthetic reactions such as purine and dTMP syntheses, formyl modification of mitochondrial  $\text{tRNA}^{\text{Met}}$ , DNA and histone methylation, lipid head group modifications and creatine metabolism. Methyl cycle and folate cycle are essential players in maintenance of the methylation capacity in a cell. See schematic representation of 1C metabolism in Figure 10. (Ducker, 2017).



**Figure 10** Simplified scheme of folate and methyl cycle interconnection and dependent cellular pathways. Red arrows and balls highlight our findings of glucose-driven induction in 1C metabolism and transsulfuration in mitochondrial myopathy muscle.

Serine is not only needed as precursor for transsulfuration reactions, as shown in Figure 9, but also serves as a major carbon donor for cellular 1C reactions through the folate cycle (Figure 10). In the folate cycle, folate, or vitamin  $\text{B}_9$ , is first transformed to tetrahydrofolate (THF). THF accepts a 1C unit in different oxidation states and is cycled in a sequence of reactions compartmentalized in the mitochondria and cytoplasm. By transcriptome analysis, we have previously reported induction of mitochondrial folate cycle enzyme, methylenetetrahydrofolate dehydrogenase (MTHFD2), in the muscle of the Deletor mice (Tynismaa, 2010). Encouraged by this, we measured folate cycle intermediates (forms of THF) and detected increased levels of 5,10-methenyl-THF and 5-methyl-THF together with high protein expression of mitochondrial enzymes MTHFD2 and MTHFD1L, suggesting major alterations in mitochondrial and cytoplasmic folate cycle.

In different cell types, or upon nutritional stress, the direction of THF-cycling reactions can be reversed to favor the availability of 1C units between the different synthetic pathways (Ducker, 2016, 2017). In Deletor muscle, we observed an increased amount of purine metabolism intermediates that together with dNTPs and transsulfuration could indicate increased need of nucleotides for mtDNA replication stress (5.1.2).

The mitochondrial folate cycle and mitochondrial translation are directly linked through formylation of the mitochondrial initiator methionine, formyl-methionine (fMet and fMet-tRNA<sup>Met</sup>). Mutations in mitochondrial methionyl-tRNA formyltransferase (MTFMT) that generates the fMet-tRNA<sup>Met</sup> compromise mitochondrial protein synthesis leading to OXPHOS deficiency (Tucker, 2011). In Deletors, mitochondrial translation assay has indicated modestly decreased organellar translation (unpublished data of ASW laboratory). In Deletor muscle, however, the large mtDNA deletions directly impair several tRNAs and affect their levels, possibly directly reducing the rate of translation in mitochondria. We do, however, detect multiple changes especially in the mitochondrial part of the folate cycle that could potentially lead to currently uncharacterized blocks or imbalances in formulation reactions, and therefore further impair the initiation of mito-translation.

Folate and methyl cycles are interdependent, linked by a folate cycle intermediate, 5-methyl-THF. In the Deletor muscle, we detected high level of 5-methyl-THF, as well as betaine, choline and threonine that can also be utilized to maintain methyl cycle (Barlowe, 1988; Christensen, 2006; Tibbetts, 2010; Ducker, 2017) (Figure 10). As shown in the previous chapters of section 5.1, our results suggested that the usage of folate cycle could be partially boosted to support methyl cycle to drive transsulfuration (Figures 9 and 10). However, methylation of homocysteine to re-generate methionine in methyl cycle is essential to maintain proper ratios of S-adenosyl methionine (SAM) and de-methylated S-adenosyl homocysteine (SAH). SAM is the major 1C donor utilized by methyltransferase enzymes, for example in synthesis of creatine, in epigenetic modification of nucleic acids and histones, and synthesis of membrane phospholipids, such as phosphatidylcholine. Increased SAH/SAM ratio is an indicator of methyl donor deficiency, and is observed in folate deficiency and defects in other vitamins essential for folate and methyl cycles (Walker, 2017). In Deletor muscle, however, SAH and SAM levels were normal and a qualitative assay of bulk DNA methylation did not show difference from healthy controls. Nevertheless, further studies on the cellular trans-methylation reactions are with high relevance for mitochondrial disease pathology, as increasing evidence have already shown the importance of methylation balance in robust epigenetic gene expression regulation and pathophysiology of neurological disorders (Gao, 2018; Zhang, 2018).

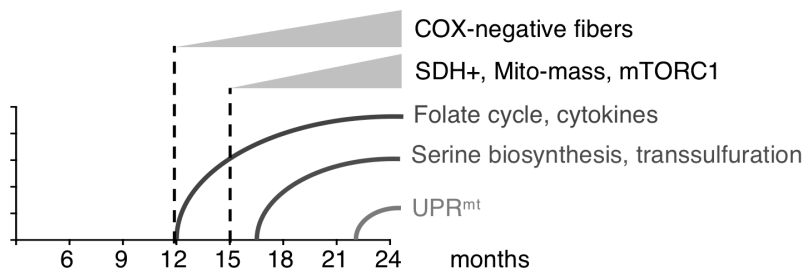


## 5.2 MITOCHONDRIAL INTEGRATED STRESS RESPONSE (ISR<sup>MT</sup>) IS CONSERVED AND SEQUENTIAL IN MAMMALS (I)

For comprehensive and unbiased characterization of the transcriptome in humans, we performed RNA-sequencing of AdPEO muscle samples. In patients with mtDNA deletions, we detected robust induction of the genes important for the metabolic reprogramming described in 5.1 (III\*). More specifically, transcripts of the mitochondrial folate pathway (*MTHFD1* and *MTHFD2*) and serine metabolism (*PHGDH*, *PSAT1* and Serine hydroxymethyltransferase, *SHMT2*) were significantly increased. Also, the key secretory hormone-like cytokines, *FGF21* and *GDF15*, were among the most significantly upregulated transcripts, replicating the transcriptional characteristics of initial AARE-response described in Deletors (Tynismaa, 2010) (see 2.5.1). The transcriptional AARE and the metabolic stress responses to mitochondrial insults in mammalian muscle are hereafter collectively termed the mitochondrial integrated stress response, or ISR<sup>mt</sup>.

Our previous studies describing the ISR<sup>mt</sup> in mitochondrial myopathy (patients and Deletor) were mainly cross-sectional, performed in the advanced stage of pathogenesis. However, expression of *Fgf21*, a key AARE-driven gene, was shown to increase along with the progression of histological respiratory chain deficiency in muscle fibers (Tynismaa, 2010). Therefore, we intended to study whether we can dissociate different phases for the ISR<sup>mt</sup> and identify the driving transcription factors.

For the dynamic characterization of ISR<sup>mt</sup>, we systematically collected Deletor tissues prior to (9 months of age) and throughout the manifesting period (12-24 months of age), and analyzed gene expression of key ISR<sup>mt</sup>/AARE factors. In the early phase of pathology, corresponding with appearance of the first detectable COX-negative fibers at 12 months of age, we detected transcriptional induction of *Fgf21*, *Gdf15* and *Mthfd2*. The first stage therefore marked initiation of systemic signaling with hormones, together with mitochondrial one-carbon metabolism. Induction of the serine biosynthetic enzymes, *Phgdh* and *Psat1*, was apparent only months later, around 18-20 months of age, after the appearance of SDH-positive fibers and activation of mTORC1 (Figure 11). The clear temporal difference between initiation of serine synthesis and the first stage AARE-genes suggests that the glucose/serine-driven metabolic rewiring is not the primary response to mitochondrial dysfunction in muscle, but could be initiated in response to, or regulated by, the first stage ISR<sup>mt</sup>.



**Figure 11** Schematic representation of the ISR<sup>mt</sup> phases and UPR<sup>mt</sup> relative to signs of disease progression in muscle of Deletor.

Mitochondrial unfolded protein response (UPR<sup>mt</sup>) has strongly featured in the discussion of mitochondrial stress signaling for the last decade. The UPR<sup>mt</sup> includes expression of numerous heat shock proteins (HSP), chaperones and proteases with undisputed mechanistic importance in mitochondrial stress signaling in *C. elegans* models of mitochondrial dysfunction (Haynes, 2007; Durieux, 2011; Nargund, 2012; Merkwirth, 2016; Zhang, 2018). In mammals, evidence for UPR<sup>mt</sup>/HSP induction in mitochondrial pathology derives from cultured cells with proteotoxic stress (Zhao, 2002) or severe mitochondrial dysfunction due to knockout of essential proteins in a tissue-specific context (Pulliam, 2014; Seiferling, 2016). In Deletor mouse muscle, we found only a modest increase in gene expression of heat shock protein 70 (*Hsp70*), and increased mitochondrial localization of HSP10, HSP60 and LONP1 proteins, but only at the very late stage of the mouse lifespan, after 22 months of age (Figure 11). Similarly, in RNA sequencing data of AdPEO patients' muscle, we did not detect induction of UPR<sup>mt</sup>/HSP markers. This data suggested that the UPR<sup>mt</sup>, as described in the worm or knockout models, is not significant in muscle manifesting mtDNA expression disorders of mammals. This suggests that the underlying molecular and biochemical events are profoundly different in different model systems and cells, causing the tissues to respond with a different combinations of stress markers.

In a *C. elegans* model of neuronal respiratory chain deficiency, the activated stress responses were described to spread from the affected tissue to distant, healthy cells through secreted molecules or 'mitokines' (Durieux, 2011) (see 2.5.1). In Deletor model, mtDNA deletions and respiratory chain deficiency is detected in muscle and neural tissues (and brown adipose tissue) (Suomalainen, 1992; Tynismaa, 2005; Khan, 2014), although the mutant Twinkle is ubiquitously expressed. To study whether the secreted ISR<sup>mt</sup> components FGF21 and GDF15 (or unknown other factors) could initiate stress responses in the 'non-affected' secondary tissues, similar to the worm, we screened gene expression of *Fgf21*, *Mthfd2*, *Hsp60* and *Hsp70* in skeletal muscle, heart, liver, adipose tissues, lungs and kidneys. Importantly, we only detected expression of *Fgf21* and *Mthfd2* in the primarily affected skeletal

muscle and heart, as well as marginal induction of *Mthfd2* in brown adipose tissue, and not in the brain or peripheral tissues. This indicated that the mitochondrial stress responses, caused by mitochondrial replicative stress and mtDNA deletions, do not get induced in secondary tissues without the primary mitochondrial defect.

### 5.2.1 ISR<sup>mt</sup>: MIX-AND-MATCH OF DIFFERENT ATFS

AARE-promoters are bound by activating transcription factors (ATFs) that drive expression of downstream genes. ATF4 and ATF5 have been predominantly studied in the context of mammalian mitochondrial insults in cell culture conditions (Fiorese, 2016, Quirós, 2017). In a cell culture model of mtDNA depletion, ATF4 was described to drive activation of ISR<sup>mt</sup> (Bao, 2016). Patient derived cell lines do not, however, usually preserve multiple mtDNA deletions in culture conditions. Therefore, to study dynamics of ISR<sup>mt</sup> and ATFs in culture we challenged mtDNA expression with reversible chemical inhibition of mitochondrial translation by actinonin. Actinonin is previously known also to induce expression of AARE-genes in culture (Richter, 2013) and is therefore mechanistically close to translation defect in a system with mtDNA deletions (e.g. the Deletor). In actinonin treated fibroblasts (mouse and human) and mouse myoblasts we showed that the components of the mammalian ISR<sup>mt</sup> (Figure 11) were robustly induced following the gradual inhibition of mitochondrial translation. Out of ATFs 1-7, only ATF3, 4 and 5 were robustly induced in transcriptional assays. RNA-interference experiments showed that out of ATFs 3-5 only ATF4 was needed for ISR<sup>mt</sup> activation *in vitro*.

To compare whether the established ATFs 3-5 of ISR<sup>mt</sup> *in vivo* were similar to culture conditions, we analyzed expression data of Deletor mouse and AdPEO patient muscle. Different to cultured cells on actinonin or mtDNA depletion model (Bao, 2016), we found ATF5 to be robustly expressed together with the ISR<sup>mt</sup> factors. We did not detect increased expression of ATF3 and ATF4 in patients, but saw their modest induction in late stage of disease progression in Deletor muscle, along with UPR<sup>mt</sup>.

In summary, we suggest that ATF5 is the primary ATF associated with ISR<sup>mt</sup> in post-mitotic mitochondrial myopathy muscle *in vivo*, different from proliferating cells in culture. Our work highlights that ATFs work in concert, depending on the type of stress, cell type or organism, and that there likely is no definite uniform driver for the stress responses in mitochondrial disease.

## 5.3 FGF21 DRIVES THE DYNAMICS OF LOCAL AND SYSTEMIC PATHOPHYSIOLOGY (I)

FGF21 was one of the first components of ISR<sup>mt</sup> to get induced in the sequence of pathology of Deletor muscle, as presented above. Importantly, FGF21 is an endocrine hormone with potential to tune the metabolism of the whole body upon chronic exposure. Therefore, we studied the local and systemic effects of chronic FGF21 signaling upon mitochondrial myopathy, and genetically inactivated FGF21 in the Deletor mouse.

### 5.3.1 GENERATION, VALIDATION AND LIMITATIONS OF THE DELETOR-FGF21 KNOCKOUT MODEL

*“Essentially, all models are wrong, but some are useful.”*

–George E. Box

The Deletor-FGF21KO mouse (DEL-FKO) was generated by sequential crossing of *Fgf21*<sup>LoxP/LoxP</sup> mice (Potthoff, 2009), ubiquitously Cre-expressing mice [PGK-Cre, (Lallemand, 1998)] and Deletors (Tynismaa, 2005). A full-body knockout of FGF21 was confirmed by lack of gene and transcript from multiple tissues, and by fasting serum FGF21 measurements. All mouse strains had normal lifespan and appearance.

Lack of FGF21 in essential metabolic organs, such as liver and adipose tissues, have been reported to compromise the systemic responses to nutrition, carbohydrate and fat metabolism (Badman, 2007, 2009; Potthoff, 2009; Xu, 2009). Therefore, the ubiquitous FGF21KO background always must be considered, especially in interpretation of the cell non-autonomous traits. Direct comparison of Deletors and DEL-FKO in metabolic assays need to be normalized to wild type or FKO background.

Second, the experimental setup using ubiquitous FGF21KO does not allow FGF21-related effects to be specifically attributable to the muscle derived FGF21. Unfortunately, a muscle-specific knockout we initially made (with ACTA1 driven Cre-recombinase, unpublished), had a significant leak of FGF21 expression from the key tissues of interest, the skeletal muscle and heart, and was therefore not suitable for this study. On the other hand, a full body knockout conveniently eliminates compensatory or interfering expression of FGF21 from secondary and subtly affected tissues, such as the brown adipose.

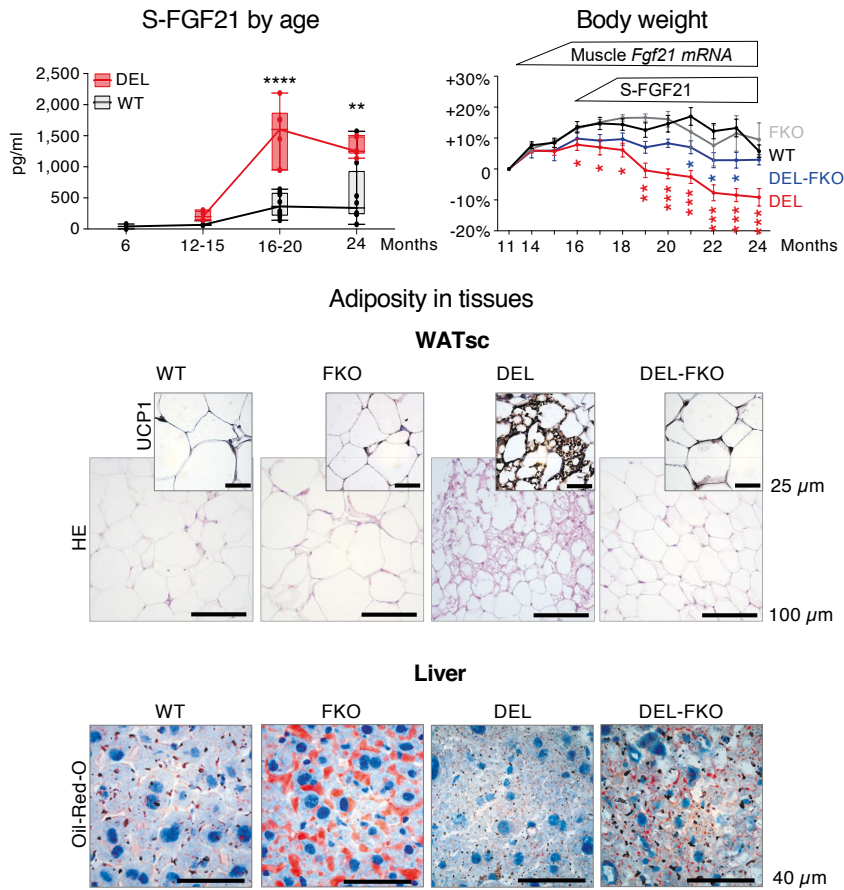
In summary, the limitations of our model disable studies on the tissue-to-tissue interactions dynamically or with mechanistic certainty. Therefore, we do not propose receptor interactions or exact molecular mechanisms for the FGF21-effects in the target tissues. However, as we have demonstrated that skeletal muscle and heart are the primary significant sources of FGF21 in the Deletor (5.2), a ubiquitous knockout of FGF21 essentially models the muscle-specific knockout set-up in laboratory animals with minimal need to initiate

physiological fasting responses and metabolic adaptation in standard maintenance conditions. Ultimately, we state that results obtained from the DEL-KO, in comparison to Deletors and healthy controls, can confidently state the dependence or independence of the phenotypic features on FGF21 in the context of mitochondrial disease.

### **5.3.2 FGF21 PREDICTABLY CAUSES BROWNING OF WHITE ADIPOSE TISSUE AND BODY WEIGHT LOSS IN DELETOR**

Circulating FGF21 has been reported to promote leanness, low adiposity and increased energy expenditure in mouse models (2.5.2). Fitting to this, Deletors at the age of disease manifestation are lean, have reduced liver fat and are resistant to diet-induced obesity (Ahola-Erkkila, 2010; Tyynismaa, 2010). The lean phenotype has been rightfully hypothesized to be mediated by muscle derived FGF21, as was described for autophagy deficient *Atg7<sup>muscle-/-</sup>* mice with robust FGF21 response and subsequent loss of adipose tissue (Kim, 2013).

In Deletors of 16-20 months of age, high serum FGF21 is detected and body mass starts to progressively decline (Figure 12). DEL-FKO mice, on the other hand, show normal body weight progression almost throughout their lives. Systemic white adipose tissue browning is a known consequence of chronic FGF21 exposure, and is shown also in mice with mitochondrial uncoupling in muscle (Keipert, 2014). Here, we show that the small adipose volume of Deletors is associated with FGF21 mediated browning, as the white adipose tissue of DEL-FKO mice appeared similar to wild type controls (Figure 12). Loss of liver fat was not completely restored by FGF21 knockout (Figure 12). This could be explained by a so far uncharacterized primary pathogenesis in the liver tissue by Twinkle mutation, or by redundant regulation of fat metabolism by unknown factors, or by metabolic hormone and mitochondrial disease biomarker GDF15 (Yatsuga, 2015; Chung, 2017).



**Figure 12** **Top:** Increase of serum FGF21 (S-FGF21) in ageing Deletors and body weight progression, presented as percentage from 11 months of age. Stars indicate significant difference of experimental group and WT. **Bottom:** Fat droplet size (hematoxylin-eosin staining) on subcutaneous white adipose tissue (WATsc) and brown/beige adipocytes indicated with uncoupling protein 1 (UCP1) immunohistochemistry. Liver stained with Oil-red-O for lipids. Statistical significance: \*= $p < 0.05$ , \*\*= $p < 0.01$ , \*\*\*= $p < 0.001$ , \*\*\*\*= $p < 0.0001$ .

GDF15 was found to be robustly induced in a mouse model of respiratory chain ablation and UPR<sup>mt</sup> induction due to knockout of an accessory subunit of the mitochondrial ribosome (CRIF1) in skeletal muscle (Chung, 2017). FGF21 was also induced, but unlike GDF15, presented no correlation with mitochondrial stress factors in expression analysis of CRIF1KO<sup>muscle</sup>. In the CRIF1KO<sup>muscle</sup> model, GDF15 caused cell-non-autonomous improvement of energy expenditure, insulin sensitivity, oxidative metabolism and lipolysis in liver and adipose tissues (Chung, 2017), similar to the effects of FGF21 in Deletor. Interestingly, the underlying insults to the mitochondrial respiratory chain are profoundly different in Deletor and CRIF1KO<sup>muscle</sup> models: Deletor manifests mtDNA deletions and respiratory chain deficiency in the form of low

activity of COX and ragged red fibers, whereas CRIF1KO<sup>muscle</sup> mice have marked depletion of respiratory chain complexes (Chung, 2017), possibly due to impaired insertion into inner membrane (Kim, 2012). These examples by us and others highlight the complexity of local stress responses (covered in chapter 5.2) that originate from mechanistically different primary insults, a concept discussed further for FGF21 and GDF15 in chapters 5.7 and 5.8.

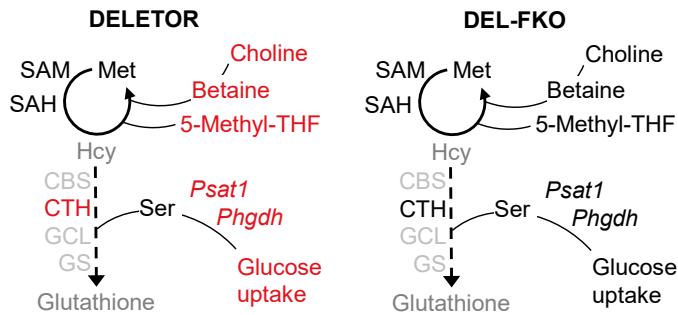
### **5.3.3 FGF21 HAS NO EFFECT ON THE PRIMARY DISEASE SIGNS OF MITOCHONDRIAL MYOPATHY**

To evaluate the effects of FGF21 on local disease progression in muscle, we first assessed the primary pathological hallmarks: mtDNA deletions, respiratory chain deficient muscle fibers and respiratory chain complexes. At 24 months of age, none of these disease signs were significantly changed in DEL-FKO relative to Deletors. We also assessed gene expression of representative ISR<sup>mt</sup>/AARE genes (*Gdf15*, *Mthfd2*, *Trib3* and *Asns*) that previously showed temporal co-expression with FGF21. Similar to mitochondrial pathology, these factors showed induction independently of FGF21. We also found that out of the ISR<sup>mt</sup>-relevant ATFs 3-5 (5.2.1), ATF5 expression was upregulated regardless of presence or absence of FGF21, whereas expression of ATF3 and ATF4 was moderately up in Deletors but did not show any induction in DEL-FKO. These results demonstrate that FGF21 does not contribute to the early disease pathogenesis, demonstrates fixed regulation and no interdependence inside the first-stage ISR<sup>mt</sup>, and further support the hypothesis of ATF5 as the mammalian driver of mitochondrial stress in muscle (5.2.1).

### **5.3.4 FGF21 IS ESSENTIAL FOR TRANSSULFURATION AND ONE-CARBON METABOLISM IN MITOCHONDRIAL MYOPATHY**

In the pathophysiology of mitochondrial myopathy muscle, glucose uptake, serine *de novo* synthesis and induction of transsulfuration marked the metabolic rearrangements in Deletor muscle (Figures 8 and 9). In the sequence of transcriptional response, FGF21 expression preceded induction of serine biosynthetic enzymes, PHGDH and PSAT1 (Figure 11). We therefore measured expression of serine synthesis enzymes and protein amount of transsulfuration enzyme CTH in DEL-FKO. Remarkably, in DEL-FKO at 24 months of age, we did not observe induction of *Phgdh*, *Psat1* or CTH, markedly elevated in Deletors. Importantly, uptake of [<sup>18</sup>F]-FDG was not induced in the affected muscle and heart tissues of DEL-FKO (see biodistribution of [<sup>18</sup>F]-FDG in Figure 14). Furthermore, we noted that in muscle of DEL-FKO mice, levels of the methyl donors 5-methyl-THF, betaine and choline, and trans-methylation intermediates guanidinoacetic acid and creatine were normal, whereas in Deletor they are induced compared to healthy controls.

Together, the metabolomics and expression data suggest that persistent induction of FGF21 upon mitochondrial dysfunction is required for the robust one-carbon metabolism rearrangements in mitochondrial myopathy, especially for the glucose driven serine synthesis and transsulfuration induction. Scheme in Figure 13 summarizes these key changes around methyl cycle and transsulfuration pathways in muscle of Deletors and DEL-FKO.



**Figure 13** Schematic presentation of the key transsulfuration and methyl cycle reactions with summary of the findings in skeletal muscle of Deletor and DEL-FKO. Color codes: Red=induced, Black=normal, Grey=not measured/not detected. Abbreviations: CBS=Cystathionine beta-synthase, CTH=Cystathionine gamma-lyase, GCL=Glutamate-cysteine ligase, GS=Glutathione synthetase, Hcy=homocysteine, Phgdh=Phosphoglycerate dehydrogenase, Psat1=Phosphoserine aminotransferase, SAH=S-adenosyl homocysteine, SAM=S-adenosyl methionine, THF=tetrahydrofolate.

### 5.3.5 SYSTEMIC GLUCOSE PREFERENCES IN MITOCHONDRIAL MYOPATHY ARE MODULATED BY FGF21

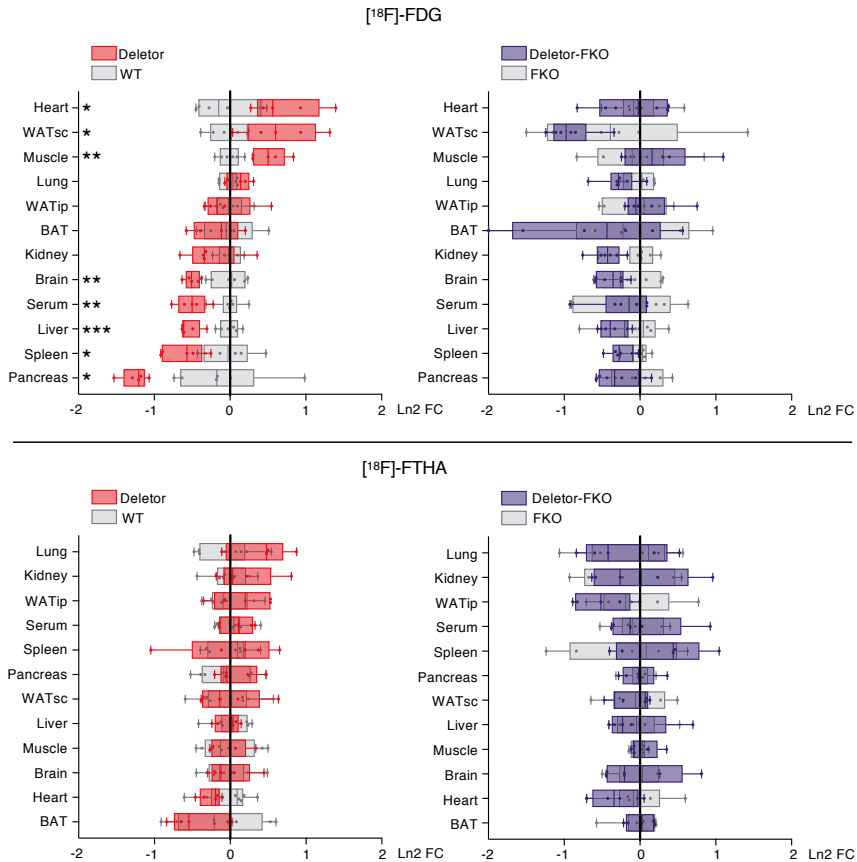
To obtain more insight into role of FGF21 in systemic nutrient metabolism, we performed *in vivo* biodistribution assays of radio-labelled glucose and fatty acid analogues, [ $^{18}\text{F}$ ]-FDG and [ $^{18}\text{F}$ ]-FTHA, respectively. First, in Deletors, the affected muscle and heart, as well as white adipose tissue with browning phenotype, showed significantly increased [ $^{18}\text{F}$ ]-FDG uptake. Moreover, several other tissues of the Deletor showed changes in glucose uptake. We observed a decrease in relative [ $^{18}\text{F}$ ]-FDG uptake in brain, liver, pancreas and spleen, and less [ $^{18}\text{F}$ ]-FDG was retained in serum of Deletors after the uptake period, possibly reflecting the increased demand of the muscle tissues. Interestingly, DEL-FKO mice had [ $^{18}\text{F}$ ]-FDG uptake comparable to FKO controls ( $p > 0.05$ ) in all the tissues we measured (Figure 14). These results show that 1) FGF21 drives glucose uptake especially in the affected muscle and heart, 2) browning of white adipose tissue is reflected as an increased metabolic activity, and 3) the systemic exposure to FGF21 potentially modulates glucose preferences of the brain and whole periphery.

On the other hand, uptake of the palmitic acid analog, [ $^{18}\text{F}$ ]-FTHA, was not changed in comparison of Deletor and wild type nor in DEL-FKO and FKO.



Given the well-known actions of FGF21 in boosting of fatty acid metabolism in rodents, it was somewhat surprising that the uptake of fatty acids ([ $^{18}\text{F}$ ]-FTHA) was not changed in the tissues of Deletor (or DEL-FKO).

In summary, these results suggest that the interplay of mitochondrial myopathy and FGF21 mainly affects glucose but not long chain fatty acid uptake in the primarily affected or the secondary tissues (Figure 14).



**Figure 14** *In vivo* glucose (upper panel) and fatty acid (lower panel) uptake assays with [ $^{18}\text{F}$ ]-labeled analogs. Deletors and DEL-FKO mice are superimposed with the appropriate healthy controls, wild type and FKO, respectively. Ln2 of fold change for SUV (Standard uptake value) presented, statistical significance: \*= $p < 0.05$ , \*\*= $p < 0.01$ , \*\*\*= $p < 0.001$ .

### 5.3.6 FATTY ACID OXIDATION AND FGF21 IN MITOCHONDRIAL MYOPATHY (I AND III\*)

In metabolic characterization of patient muscle and serum, we demonstrated profound increase in amino-acid and acyl-carnitine pools, and the profile was similar in the Deletor muscle (III\*). Interestingly, in publication I, we showed

that in DEL-FKO muscle, levels of long-chain acyl-carnitines were still elevated, suggesting that FGF21 was not the reason for accumulation in the affected muscles.

Acyl-carnitines are fatty acid molecules attached to a carnitine carrier, committed for beta-oxidation in mitochondria (see 2.1.2), and also utilized as markers of fatty acid oxidation disorders (Sim, 2002). Accumulation of acyl-carnitines in muscle of AdPEO and Deletor therefore suggest altered utilization, or partially blocked beta-oxidation in the muscles with mitochondrial dysfunction. Indeed, impaired fatty acid oxidation is a well-known secondary manifestation of respiratory chain deficiency. Mechanistically the disturbance can link to disbalanced NAD<sup>+</sup>/NADH pools (see 2.1.2), and indirect evidence suggests that deficient mitochondrial beta-oxidation can disturb mitochondrial membrane potential and therefore critically impair assembly and function of the mitochondrial respiratory chain (Lim, 2018). These interesting questions on causative relations OXPHOS and beta-oxidation in mtDNA instability are not mechanistically answered by our studies, but suggest involvement of fatty acid metabolism in the primary pathophysiology and stress signaling, see discussion below.

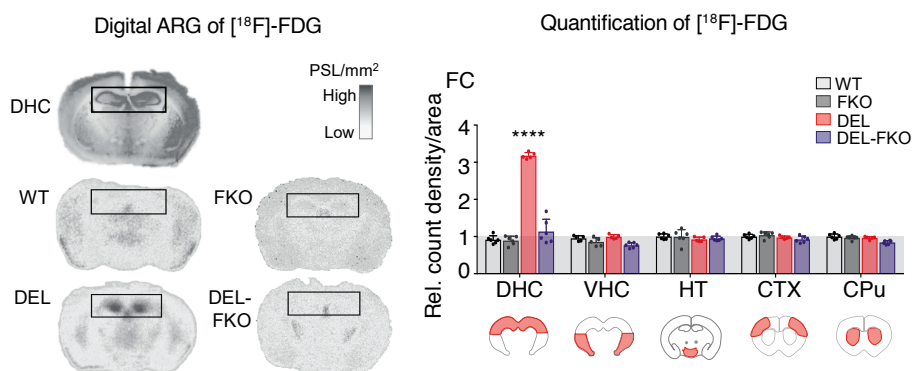
Previous study from our group and two examples from the fatty acid oxidation field suggest shared stress responses. We have shown that in the respiratory chain deficient fibers of Deletor mTORC1 is activated and drives the expression of FGF21/AARE and the one-carbon metabolism changes (Khan, 2017). Interestingly, in two models of muscle or heart specific fatty acid oxidation deficiency, an essentially identical induction of metabolic stress signaling is described. A *Cpt1b*<sup>Muscle/-</sup> mouse with inhibition of acyl-carnitine transport over mitochondrial membrane (Vandanmagsar, 2016) and a *AcsL*<sup>Heart/-</sup> mouse with impaired cytoplasmic conversion of long-chain fatty acids to acyl-CoAs (Schisler, 2015) both activate mTORC1 and present a profound increase in expression of FGF21. The response in the *AcsL*<sup>Heart/-</sup> model impressively demonstrates determinative expression of several AARE-genes: *Fgf21*, *Gdf15*, *Mthfd2*, *Trib3* and *Asns*. Moreover, the *AcsL*<sup>Heart/-</sup> even presents increased drive towards cysteine and glutathione synthesis through transsulfuration. These examples spark an interesting hypothesis for beta-oxidation as potential scanning point for mitochondrial (dys)function and induction of the ISR<sup>mt</sup>. Therefore, the initial signal for FGF21/AARE response might originate from the primary or secondary impairment of beta oxidation and energy metabolism, upon which induction of FGF21 seems physiologically relevant.

## 5.4 FGF21 MEDIATES MITOCHONDRIAL PATHOLOGY IN CA2-REGION OF HIPPOCAMPUS (I)

FGF21 is a known regulator of metabolism, acting on the interplay between the periphery and hypothalamus (2.5.2 and 2.5.3). Based on the literature and widely altered biodistribution of [ $^{18}\text{F}$ ]-FDG in Deletors (Figure 14), we hypothesized that chronically high FGF21 in the circulation could influence the brain.

### 5.4.1 FGF21 DRIVES GLUCOSE UPTAKE IN THE CA2

Parallel to the *in vivo* biodistribution studies (Figure 14), we performed autoradiography of brain sections after [ $^{18}\text{F}$ ]-FDG uptake. From the series of coronal sections, we identified an intense and specific [ $^{18}\text{F}$ ]-FDG signal, located in the dorsal hippocampus (DHC) of Deletors. We quantified the [ $^{18}\text{F}$ ]-FDG signal of multiple brain regions, including the dorsal and ventral hippocampus, hypothalamus, cortex and striatum, and only found change in the Deletor DHC. Strikingly, FGF21 was required for the intense glucose uptake to the DHC of Deletors, as [ $^{18}\text{F}$ ]-FDG signal in the DHC of DEL-FKO brain was similar to healthy controls (Figure 15).

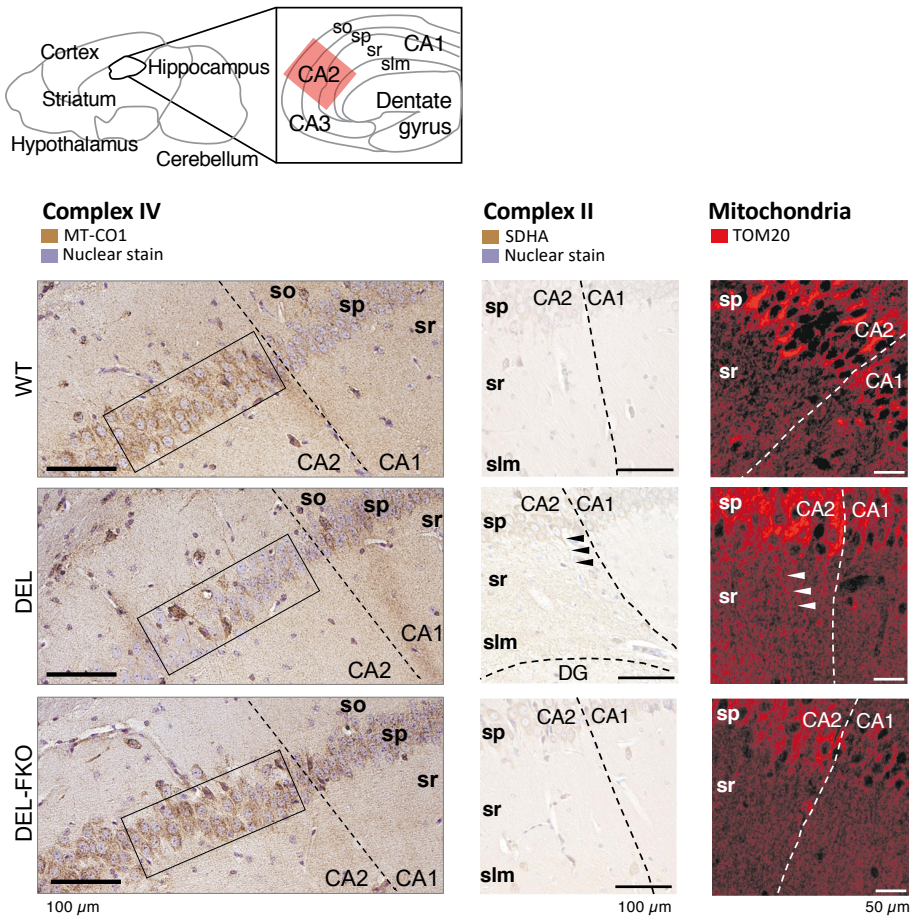


**Figure 15** Digital autoradiography of coronal brain sections in DHC and quantitation of [ $^{18}\text{F}$ ]-FDG intensity in different brain regions (PSL=photostimulated luminescence). Statistical significance: \*\*\*\*= $p < 0.0001$ . Abbreviations: DHC=Dorsal hippocampus, VHC=ventral hippocampus, HT=hypothalamus, CTX=cortex (visual), CPu=caudoputamen of striatum.

### 5.4.2 FGF21-DEPENDENT MANIFESTATION OF MITOCHONDRIAL PATHOLOGY IN THE CA2

Previously, Deletors were known to harbor distinct COX-/SDH+ cell populations in purkinje cell layer of cerebellum and hippocampus (Tyynismaa, 2005). Guided by the increased glucose uptake, we now focused on the Deletor DHC and demonstrated a depletion of Complex IV (MT-CO1) and

mitochondrial proliferation (SDHA and TOM20), classical signs of respiratory chain deficiency in muscle. Intriguingly, mitochondrial respiratory chain deficiency in Deletors was strictly localized to the CA2 sub-region of the hippocampus, and completely absent in the neighboring hippocampal regions CA1, CA3 and dentate gyrus, as well as extra-hippocampal regions such as the cortex and hypothalamus. Most importantly, FGF21 was indispensable for MT-CO1 depletion and mitochondrial proliferation in the CA2, as DEL-FKO mice were indistinguishable from the healthy controls (Figure 16), as was the [ $^{18}\text{F}$ ]-FDG uptake presented before (Figure 15).



**Figure 16** **Top:** Schematic presentation of mouse brain in sagittal orientation with a detailed map of hippocampus divided to major sub-regions and layers. **Bottom:** Immunodetection of mitochondrial respiratory chain Complex IV (MT-CO1, mtDNA encoded), Complex II (SDHA, nuclear encoded) and mitochondrial outer membrane protein (TOM20). Visuals: boxes indicate the sp-layer of CA2 in MT-CO1 staining, arrows point to SDHA and TOM20 positive neuronal projections of sr-layer. Abbreviations: DG=dentate gyrus, so=stratum oriens, sp=stratum pyramidale, sr=stratum radiatum, slm=stratum lacunosum-moleculare.

In Deletors, mtDNA deletions underlie the respiratory chain dysfunction in the muscle, heart and brown adipose tissue (Tynismaa, 2005; Khan, 2014, III\*). The DHC (containing most of the anatomical CA2), VHC and cerebellum showed equal amounts of mtDNA deletions in Deletors and DEL-FKO, similar to the skeletal muscle. This indicates that the metabolic pathology (glucose uptake and respiratory chain deficiency) in CA2 did not affect the primary mtDNA mutagenesis in Deletor background.

To study whether FGF21 alone influenced glucose metabolism of the different brain areas, we analyzed [<sup>18</sup>F]-FDG uptake in wild type mice that were either injected with recombinant mouse FGF21 or fasted for 24 hours to induce the physiological starvation response with hepatic expression of FGF21. As a result, [<sup>18</sup>F]-FDG uptake was increased in the whole brain of fasted mice, but hippocampus, hypothalamus or cerebellum were not affected by fasting or recombinant FGF21.

These results indicate that FGF21-dependent mechanisms modify the glucose metabolism and mitochondrial pathology specifically in the CA2 of hippocampus expressly in the context of primary mitochondrial dysfunction (mtDNA deletions).

#### **5.4.3 FGF21 LINKS THE CA2 TO NON-HOMEOSTATIC METABOLIC REGULATION?**

The Ammon's horn of hippocampus consists of CA1, CA2, C3 and dentate gyrus. Of the CA-regions, CA2 was the last to be identified and its role is only recently being elucidated in the canonical hippocampal circuits; spatial processing, social behavior and temporal encoding (Dudek, 2016; Tzakis, 2019). Structurally, CA2 differs from the neighboring CA-regions significantly. Even on a simple histology, CA2 structure is defined by presence of large and loosely packed pyramidal neurons, different from the neighboring CA1/CA3. Expression of specific markers, such as purkinje cell protein 4 (PCP4) and regulator of G protein signaling (RGS14), indisputably identify CA2 (Dudek, 2016; Gerber, 2019).

CA2 has attracted great interest in neurobiology as it has shown to present superior resistance to damage by injury (Dudek, 2016). Calcium ion buffering capacity and extrusion rates are nearly four times greater compared to neighboring CA1, and for example a robust calcium transporter of inner mitochondrial membrane ryanodine receptor 1 (*Ryr1*) is highly expressed in CA2 (Jakob, 2014). Mitochondria are central in cellular calcium metabolism, and along with enrichment of calcium factors, mitochondrial transcripts are generally overrepresented in transcriptome of CA2 (Farris, 2019). Furthermore, availability of glucose has been hypothesized to maintain sufficient calcium buffering and memory formation in hippocampus, with implications in Alzheimer's disease (Holahan, 2019). Together, these independent results from other fields associate our results on FGF21 dependent glucose uptake and mitochondrial regulation in CA2 in a frame

where FGF21 serves important functions in maintaining adaptive responses in central nervous system, especially upon mitochondrial pathology.

In the literature, CA2 has emerged with connections to hypothalamus. The most prominent extra-hippocampal connectivity of CA2 are directly with paraventricular nucleus of hypothalamus (PVN) and supramammillary nucleus of hypothalamus (SUM) (Cui, 2013; Kohara, 2014). Interestingly, SUM can be found to express FGF21 as the ninth most upregulated gene relative to other brain areas [*Harmonizome*-database (Rouillard, 2016)]. Furthermore, systemically administered FGF21 has been directly shown to activate neurons of the PVN (Santoso, 2017; Matsui, 2018).

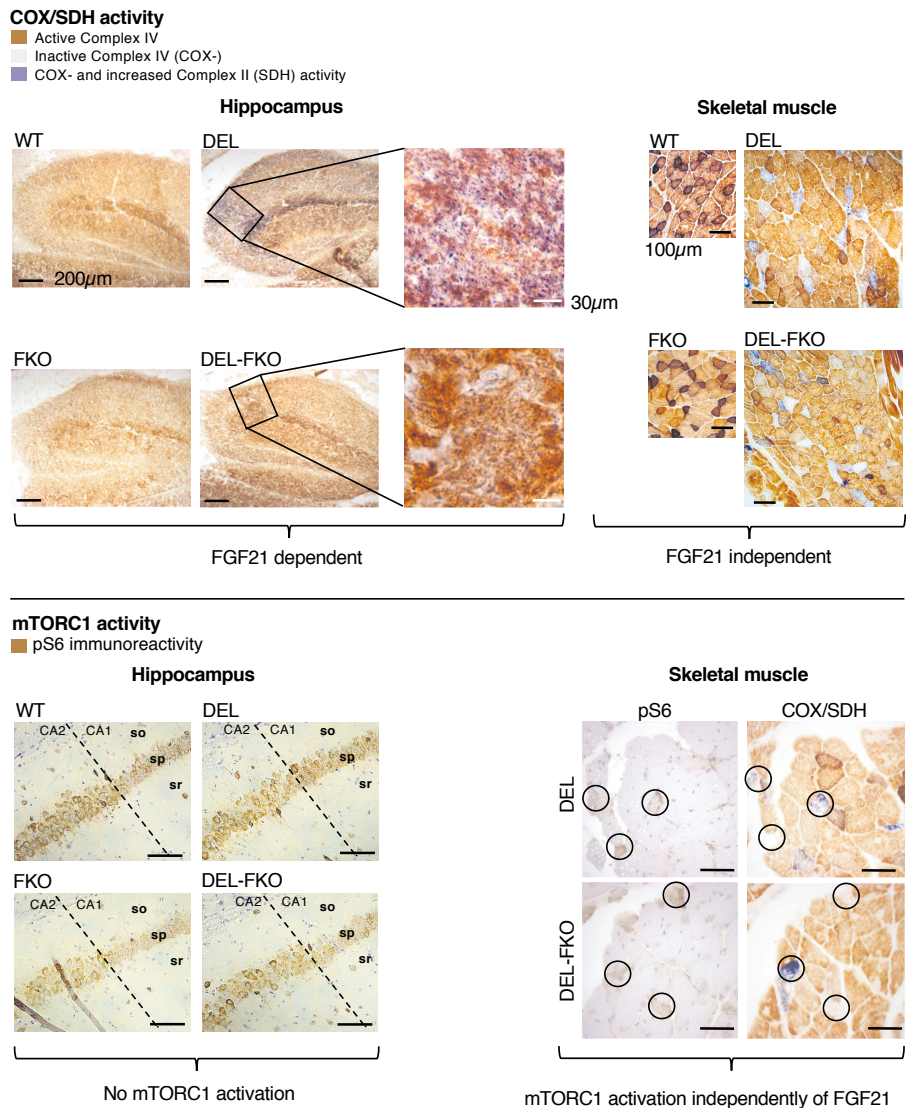
Metabolic hormones from the periphery are known to modify synaptic messaging and hypothalamic regulation of metabolism indirectly through other brain areas, such as hippocampus and cortex (comment in Maffei and Mainardi 2019). Interestingly, such mechanisms have indeed been reported for GDF15, another hormonal factor induced as part of the ISR<sup>mt</sup>. GDF15 was shown to bind to extra-hypothalamic receptors, through which the hormone mediates regulation of metabolism, important in adaptation to non-homeostatic stressful conditions (Hsu, 2017). With playful thinking, these findings spark interesting speculation whether the hypothalamic regions with FGF21 expression or receptors for peripheral FGF21 could mediate for example behavioral or other yet uncharacterized adaptive responses via the hippocampal CA2.

## **5.5 FGF21 MARKS THE TISSUE-SPECIFIC MANIFESTATION OF RESPIRATORY CHAIN DEFICIENCY CAUSED BY MTDNA DELETIONS (I AND III\*)**

In summary, previous results and data presented in this thesis show that the ubiquitously expressed dominant Twinkle mutant protein causes mtDNA mutagenesis specifically in the skeletal muscle, heart and brain of Deletor mouse (and AdPEO patients). In this thesis, we demonstrate that the mtDNA deletion pathology in the brain and muscle differ in many aspects, especially in relation to FGF21-signaling. Overall, these key differences unravel new tissue-specific features for Twinkle and mtDNA deletion pathophysiology, and highlight the importance of secondary messengers even as potential drivers of the mitochondrial deficiency. See the following bullet-points for the summary:

- FGF21 and other transcriptional components of AARE/ISR<sup>mt</sup> are induced in muscle tissues but not in the brain of Deletor. Moreover, the metabolic fingerprints are not similar in the muscle and (dorsal) hippocampus of Deletors. Especially in hippocampus, however, the heterogeneity of the cell populations challenges the bulk analysis of metabolome.

- mTORC1 activation is a distinctive hallmark of pathogenesis in the muscle but not in the brain (Figure 17).
- FGF21 does not affect the marks of respiratory chain deficiency of the muscle but completely shapes the mitochondrial and metabolic response in CA2 (Figure 17).



**Figure 17** Comparison of respiratory chain activity (top), mTORC1 activation (bottom) in muscle and brain. The effect of FGF21 for those readouts concluded for the CA2 of hippocampus and skeletal muscle.



### 5.5.1 FGF21, FOR BETTER OR WORSE?

Deletor is a physiological model of mitochondrial myopathy with a relatively mild progressive phenotype and normal lifespan. So far, the only robust behavioral phenotype is increased motor activity in treadmill (Tyynismaa, 2005). Other functional markers for scoring the phenotype are not available, and the assessment of pathology is based on histological and molecular signs, primarily mtDNA deletions and respiratory chain deficiency. For example, in a recent treatment study performed with Deletor, inhibition of mTORC1 by rapamycin brought down the ISR<sup>mt</sup>, including FGF21, and fundamentally decreased COX-/SDH+ fibers and mtDNA deletion load in skeletal muscle, interpreted as improvement of the phenotype (Khan, 2017). For FGF21 alone, we report more subtle fine-tuning of the advanced stage metabolic rearrangements of the mitochondrial myopathy muscle (5.3). In the CA2 of Deletors, on the other hand, Complex IV amount was - surprisingly - completely dependent on FGF21 presence, suggesting FGF21-mediated pathology (5.4). These findings call for further studies on pathological mechanisms, suggesting contribution of both mtDNA deletions and metabolism in the manifestation of OXPHOS deficiency.

FGF21 has reported associations in regulation of mitochondrial biogenesis and fitness that are suggested to be mediated through activation of PGC1 $\alpha$  (peroxisome proliferator-activated receptor c co-activator 1 $\alpha$ ) for example in cultured dopaminergic neurons (Mäkelä, 2014) and in the liver (Potthoff, 2009). In simplified terms, PGC1 $\alpha$  is a master regulator of responses that induce activity of mitochondria (Fernandez-Marcos, 2011), where protection of cellular fitness in various stresses involve mitochondrial biogenesis, boosting of respiratory capacity and ROS metabolism (Austin, 2012). In Deletor, treatment trials with ketogenic diet and B3-vitamin supplementation both increased mitochondrial biogenesis and improved the muscle pathology (Ahola-Erkkilä, 2010; Khan, 2014). Additionally, a direct PGC1 $\alpha$  activation strategy with bezafibrate treatment improved the muscle pathology in isolated Complex IV deficiency mouse model, Surf1KO (Viscomi, 2011), and in the Deletor (Yatsuga, 2012), but bezafibrate also caused adverse effects on the liver in Deletor. Furthermore, although mitochondrial proliferation and mtDNA copy number variation are not directly analogous events, increasing the mtDNA quantity, even with a pathologically high proportion of mutated mtDNA (>75%), was shown to alleviate the molecular pathogenesis of a mitochondrial translation and OXPHOS deficiency mouse model (Filograna, 2019).

On the contrary, evidence of FGF21-mediated deleterious effects in context of detrimental mitochondrial dysfunction has been reported. Conditional OPA1 knockout in muscle (OPA1KO<sup>muscle</sup> mouse) results in severe muscle atrophy, rapid body weight loss and death by 100 days of life (Tezze, 2017). Comprehensive knockout of OPA1 is lethal in embryogenesis (Davies, 2007), emphasizing the essential nature of the protein. In OPA1KO<sup>muscle</sup> mouse, the FGF21 response was described to be deleterious through promotion of



systemic inflammation, since knockout of FGF21 in the OPA1KO<sup>muscle</sup> background ameliorated the phenotype and prolonged lifespan (Tezze, 2017). As mentioned, in Deletor mouse the lifespan is normal and functional effects of the pathological mutation are mild but we have not detected worsening of locomotion or physical condition in the DEL-FKO mice, and we have not detected signs inflammation. The different outcomes could be explained by the outstanding difference in severity of the mitochondrial insult in the OPA1KO and the Deletor mice. For example, the earlier fatality of FGF21 expressing OPA1KO could simply lie also in complete shutdown of mitochondria and severe muscle loss in combination with significant weight loss and altered energy metabolism due to systemic actions of FGF21.

## **5.6 FGF21 AND GDF15 ARE CLINICALLY RELEVANT PROTEIN BIOMARKERS FOR MITOCHONDRIAL MYOPATHIES (II)**

Our research group was first to characterize FGF21 as a circulating diagnostic biomarker for mitochondrial diseases (Suomalainen, 2011), and others have replicated the finding in mitochondrial myopathies (Davis, 2013; Koene, 2014; Fujita, 2015; Yatsuga, 2015). Later on, also GDF15 emerged as mitochondrial disease biomarker (Fujita, 2015; Yatsuga, 2015). We aimed to review the specificity and sensitivity of the two protein biomarkers simultaneously in mitochondrial and non-mitochondrial diseases and conditions.

In clinically relevant retrospective analysis of patient samples, we found that among muscle manifesting diseases, both FGF21 and GDF15 were able to identify mitochondrial disorders from non-mitochondrial myopathies, with specificity and sensitivity exceeding the performance of conventional serum biomarkers such as lactate, pyruvate, or creatine kinase (Suomalainen, 2011). However, serum GDF15 showed generally high values among multiple secondary non-mitochondrial conditions, including cardiomyopathy, biliary cirrhosis, primary sclerosing cholangitis, metastasizing colorectal cancer and nonagenarians. This was not surprising, as GDF15 is known to be induced in a spectrum of conditions and considered as metabolic biomarker of generalized mortality (Corre, 2013). FGF21 levels also showed single high values for the metastasizing colorectal cancer cases, likely indicative of secondary liver pathology (Dushay, 2010; Yilmaz, 2010).

Overall, our results were in line with the original publications, and considered both markers useful in clinical diagnostics upon suspected mitochondrial disease, but also highlighted considerations regarding comorbidities amongst the heterogeneous population of adult patients.

## 5.7 INDUCTION OF FGF21 AND GDF15 SUGGEST CLINICALLY RELEVANT MITOCHONDRIAL TRANSLATION DEFECT (II)

We noticed that a minority of mitochondrial disease patients in our cohort showed low FGF21 and GDF15 values, despite having a confirmed mitochondrial disease diagnosis. This raised the hypothesis that specific types of mitochondrial dysfunction might elicit different responses. Those patients with low levels of biomarkers manifested diseases with underlying mutation directly affecting the structure of the respiratory chain, whereas majority of the patients with high FGF21 values had mutations affecting the maintenance and expression of mtDNA. Therefore, we wanted to more systematically review the mechanism of induction for FGF21 and GDF15 in mitochondrial diseases and conducted a composite study of meta-analysis (based on: Davis, 2013; Suomalainen, 2011; Yatsuga, 2015) and retrospective measurements in mitochondrial patients. Additionally, six mouse models with different types of representative mitochondrial defects were studied to confirm the findings.

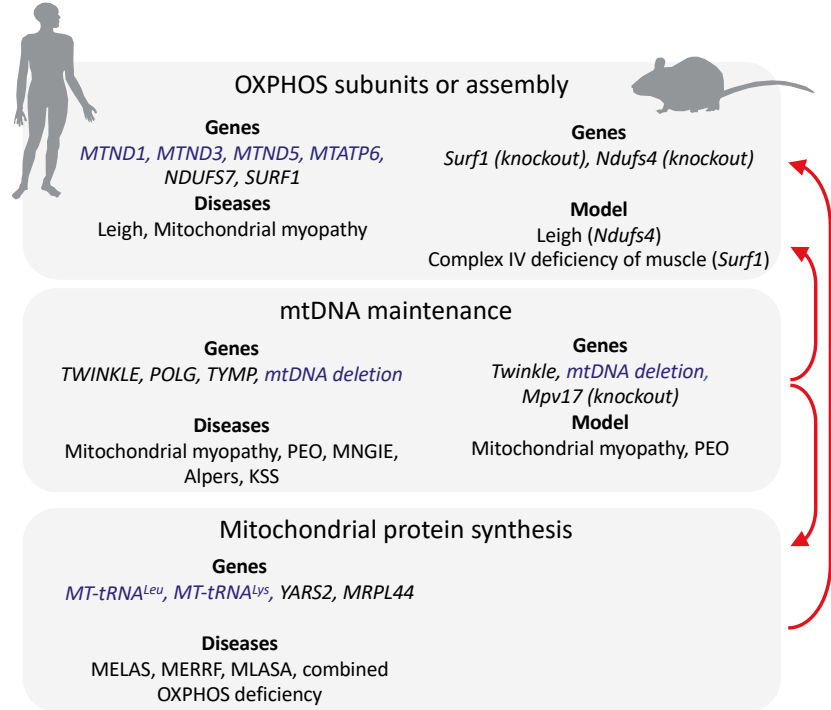
### 5.7.1 CLINICAL CHARACTERISTICS OF THE STUDY SUBJECTS

According to the best of our knowledge, primary manifestations in the central nervous system caused by mitochondrial dysfunction, without muscle involvement, do not induce an FGF21 response detectable in the serum of patients. In mice, however, a severe disruption of mitochondrial fission through *Drp1* ablation in neurons was reported to induce FGF21 in the central nervous system (Restelli, 2018). For clarity, all patients included to the study had a genetic diagnosis and muscle-manifesting (biochemical, molecular or histological) mitochondrial defect.

We then further divided the mitochondrial myopathy patients into three groups, according to their underlying genetic causes: 1) isolated RC subunit or assembly factor defects, 2) mtDNA maintenance defects and 3) mitochondrial protein synthesis defects (Figure 18). The most prevalent diseases of these three groups were Leigh disease, PEO/mitochondrial myopathy and MELAS, respectively. For more detailed description of the diseases, see chapter 2.4.

The mouse models were chosen to represent similar mechanisms and follow the same criteria for muscle manifestation (Figure 18). For primary OXPHOS deficiency, we studied samples from two well characterized models: knockout of nuclear encoded *Ndufs4* (Kruse, 2008) and *Surf1* (Dell'Agnello, 2007) that compromise Complex I activity and Complex IV assembly, respectively. The *NDUFS4*KO mouse mimics the phenotype of Leigh patients with bilateral brain lesions and premature death. In humans, *SURF1* mutations also cause Leigh (2.4.2), but the knockout mice show no neurodegeneration and live longer than wild type mice, although the Complex IV deficiency is evident in the muscle tissue. For mtDNA maintenance diseases, we analyzed mice with Twinkle mutations: the Deletor (Tyynismaa,

2005) and a mouse expressing a Twinkle point mutation (K320E) only in the skeletal muscle (Baris, 2015), both manifesting with multiple mtDNA deletions and mosaic respiratory chain deficiency in the skeletal muscle. We also included Mitomice, harboring a single, heteroplasmic mtDNA deletion (Inoue, 2000). MPV17 is a mitochondrial inner membrane protein with disease causing mutations underlying hepatocerebral mtDNA depletion disease (Spinazzola, 2006). The MPV17 knockout mouse manifests a kidney disease and significant depletion of mtDNA in the muscle, with 5-6 percentage of COX-negative muscle fibers (Spinazzola, 2006; Viscomi, 2009). At the time of our biomarker study, no mouse model of primary mtDNA point mutations were available, e.g. mimicking the common tRNA-related diseases like MELAS or MERRF. Rather recently, a mouse with a heteroplasmic mtDNA mutation in tRNA<sup>Ala</sup> (not a patient mutation) was described, manifesting in the muscle with a mild defect in mitochondrial translation and respiratory chain (Kauppila, 2016), but measurements of *Fgf21* or other stress response genes have not been reported.



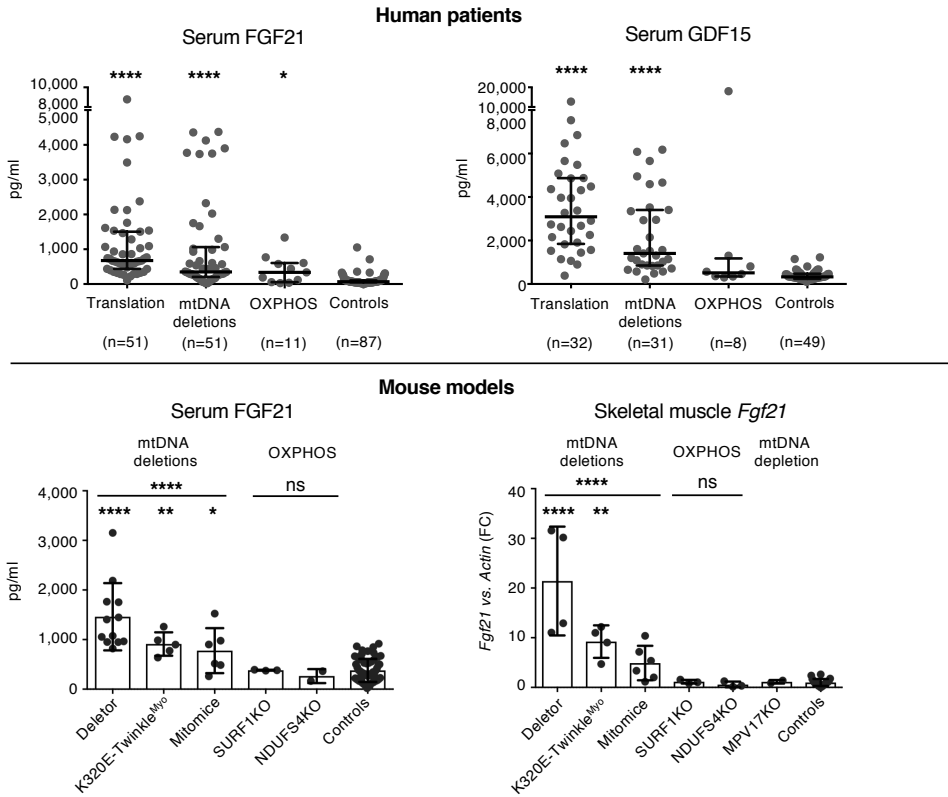
**Figure 18** Outline of the diseases and genetic causes that represent the three categories of mitochondrial myopathy in publication II. Visuals: red arrows on the right-hand side illustrate secondary causative relationship of the defects, blue text indicates primary mutation in the mtDNA.

### 5.7.2 FGF21 AND GDF15 ARE INDUCED UPON COMPROMIZED MTDNA MAINTENANCE AND MITOCHONDRIAL TRANSLATION DEFECTS

The samples from patients and mouse models analyzed in this study shared a similar pattern for serum FGF21 and GDF15. We discovered that both biomarkers were high in circulation of subjects with mitochondrial protein synthesis and mtDNA maintenance diseases. Patients and mice with mutations (or knockout) that directly affect the respiratory chain had biomarker levels comparative to healthy controls (Figure 19).

From the mouse models, we also analyzed *Fgf21* expression in the affected skeletal muscle. In mice with mtDNA deletions (Deletor, Twinkle<sup>K320E</sup> and Mitomice), expression of *Fgf21* was induced in skeletal muscle, whereas the single respiratory chain complex mutants (SURF1KO and NDUFS4KO) and mtDNA depletion model (MPV17KO) showed normal expression (Figure 19). In the muscles of Mitomice, the determined mtDNA deletion load (percentage of molecules with deletions) positively correlated with *Fgf21* expression and serum FGF21.

In summary, our results indicated that among mitochondrial myopathies, a primary or secondary mitochondrial protein synthesis defect is needed for induction of FGF21 and GDF15.



**Figure 19** **Top panel:** Serum FGF21 and GDF15 levels in mitochondrial disease patients, median and interquartile range presented with whiskers. **Bottom panel:** Serum FGF21 and skeletal muscle *Fgf21* expression in mouse models, average and standard error of mean presented with bar and whiskers. Statistical significance: \*= $p<0.05$ , \*\*= $p<0.01$ , \*\*\*= $p<0.001$ , \*\*\*\*= $p<0.0001$ .

As mentioned earlier, physiological mouse models for MELAS and other primary mtDNA protein synthesis diseases still do not exist, and therefore parallel assessment of stress responses and mechanism of molecular and biochemical pathology was not feasible. A large proportion of mitochondrial translation diseases are, however, caused by mutations in nuclear encoded mitochondrial aminoacyl tRNA synthetases (mt-aaRSs). Encephalopathy is the most common manifestation of human mt-aaRS syndromes, but heart and skeletal muscle specific knockout of *Dars2* (mitochondrial aspartyl-tRNA synthetase) is a technical model for severe mitochondrial translation deficiency of non-neuronal tissue (Dogan, 2014). Importantly, in the heart of *Dars2*KO mouse, robust induction of expression and secretion of FGF21 precedes the histological hallmarks of mitochondrial dysfunction. These results agree with ours and support the conclusion that mtDNA translation or expression deficiency induces the stress response involving FGF21 induction.

Mitochondrial translation diseases are categorically respiratory chain disorders, but the complexity and comprehensiveness of the cellular responses goes far beyond functions needed for mitochondrial protein synthesis and OXPHOS inside mitochondria (Suomalainen, 2018). In clinical context, our results and conclusions of primary or secondary mtDNA expression defect as inducer of FGF21 and GDF15 are valuable but the molecular and biochemical mechanisms remain to be studied further. In the lack of robust mouse models for mitochondrial translation diseases, *in vitro* settings have served the purpose. As an example, effects of two different chemical inhibitors of mitochondrial translation are discussed here below.

Mitochondrial translation shares many ancestral features with prokaryotic systems, leaving the mitoribosomes vulnerable to some commonly used antibiotics, relevant in clinics where routine treatment might cause unexpected adverse effects (Singh, 2014). Actinonin is a small molecule inhibitor of mitochondrial protein synthesis. Depletion of mitochondrial translation products by actinonin is followed by significant detrimental consequences for cellular fitness, as evidenced by a loss of membrane potential and reversible fragmentation of mitochondrial network (Richter, 2013, 2015). Richter et al. demonstrate that the compromised mitochondrial fitness and protein synthesis by actinonin are caused by impaired turnover or translation products, leading to accumulation of mistranslated mitochondrial polypeptides in the inner mitochondrial membrane (Richter, 2015, 2019). Interestingly, the detrimental effects of actinonin on mitochondrial fitness can be prevented by blocking protein synthesis by another drug, chloramphenicol. Chloramphenicol blocks the A-site on the mitochondrial ribosome, preventing mitochondrial protein synthesis, and therefore, double exposure prevents

elongation and essentially insertion of mistranslated products into the membrane. Importantly, the mitochondrial network and cellular proliferation are preserved in chloramphenicol, and FGF21 together with other ISR<sup>mt</sup> genes do not get induced, whereas actinonin ignites a strong stress response from the nucleus (Richter, 2013; chapter 5.2.1). This mechanism could be seen mechanistically relevant for mitochondrial disease models and patients. For example, in a cell-culture model system for MERRF mutation (homoplasmic 8344A>G in *MT-TK*), stalling of translation machinery on lysine codons due to lack of mitochondrial tRNA<sup>Lys</sup>, leads to accumulation of an aberrant mitochondrial translation product (*MT-CO1*) (Enriquez, 1995; Richter, 2015), and in MERRF patients high amount of FGF21 is seen in serum (Su, 2012).

After discussing these concepts, the mechanistic trigger for ignition of FGF21 signaling in mitochondrial protein synthesis defects (primary or secondary) could be hypothesized to involve mitochondrial membrane stress or remodeling. However, the multiple steps between the primary mitochondrial stress and the induction of nuclear stress responses (involving systemic signaling) remain unknown, and require more mechanistic research in the future.

## 6 CONCLUSIONS

Mitochondrial diseases are metabolic disorders presenting with extreme variability of tissue-specific consequences. The reasons for those manifestations cannot be explained merely by genetic causes or impaired function of OXPHOS and consequent lack of ATP. Studies of this thesis have utilized valuable patient material and disease models to shed light into pathophysiology of mitochondrial diseases by characterizing the stress responses in the primarily and secondarily affected tissues. Moreover, to study the effects of FGF21 in mitochondrial disease context, a new Deletor-FGF21KO mouse model was generated and characterized in this thesis.

In the first part of this thesis, we described a novel, well-conserved metabolic fingerprint for mitochondrial myopathy caused by mtDNA maintenance defects. In summary, the affected muscle tissues of AdPEO patients and Deletor mouse presented with remodeling of one-carbon, folate and nucleotide metabolism, as well as induction of glutathione synthesis in transsulfuration. Importantly, *in vivo* glucose uptake and flux analyses showed that the induction of one-carbon metabolism and transsulfuration were dependent on glucose-driven serine *de novo* synthesis in Deletor muscle and heart. These mechanisms that typically mark the survival of proliferative cancer were a completely novel feature for the post-mitotic muscle with mtDNA instability and mitochondrial dysfunction.

Second, we showed that the ISR<sup>mt</sup>, comprising of the transcriptional and metabolic programs, progresses sequentially in interdependent stages, and likely involves regulation by several ATFs. The first-stage ISR<sup>mt</sup> co-appeared with the first respiratory chain deficient fibers, and included expression of FGF21 and GDF15, mitochondrial folate cycle enzyme MTHFD2 and regulatory transcription factor ATF5. According to expression of PHGDH and PSAT1 enzymes, the induction of glucose-driven serine synthesis and transsulfuration initiated months after the first stage, only after advanced mitochondrial pathology was detected in the muscle (ragged-red fibers). Importantly, we showed that in the absence of FGF21 (Deletor-FGF21KO mice), no induction of serine biosynthesis, transsulfuration or methyl cycle rearrangements occurred. However, the molecular and histological hallmarks of muscle pathology or the first-stage ISR<sup>mt</sup> were not modified by presence or absence of FGF21. This suggested that induction of the first-line ISR<sup>mt</sup> markers, namely FGF21, is important for the metabolic regulation in the mitochondrial myopathy muscle.

The normal physiological role for FGF21 is to serve transient metabolic rewiring to sustain low-nutrient conditions, for example. When chronically expressed from the affected muscles upon mitochondrial disease, the physiological consequences for this hormone remain unclear. Understanding the local and systemic regulation of energy metabolism by such 'myokines' is

of high importance e.g. when dietary interventions are considered in patients. Our results on Deletor-FGF21KO model suggested that the chronic FGF21-response had different outcomes depending on the target tissue. Systemically, FGF21 caused loss of adiposity and browning of the white adipose. Moreover, the tissue-level glucose preferences were widely altered in the brain and periphery of the Deletor. Additionally, we encountered a completely novel manifestation for mtDNA maintenance defect in the central nervous system that showed undisputed dependence on FGF21. First, we showed that the ubiquitous expression of mutated Twinkle causes uniform mtDNA deletion formation the brain of Deletor, regardless of FGF21 status. Yet, specifically only the CA2-region of hippocampus manifested mitochondrial pathology coupled to intensive glucose uptake, both of which were completely FGF21-dependent. Essentially, the regulation of pathological manifestation by FGF21 was different in the muscle and the brain. Therefore, our results reveal tissue-specific regulation of mitochondrial metabolism designated by FGF21. Further studies utilizing the Deletor and Deletor-FGF21KO models are needed to understand the non-homeostatic, tissue-specific pathophysiology and regulation of energy metabolism by FGF21.

Transcriptomic analysis of human and mouse samples (as well as cell-culture experiments) showed that in mtDNA deletion disorders and mitochondrial translation defects, importance of the classical UPR<sup>mt</sup>-response was minor compared to the ISR<sup>mt</sup>. This suggests that the well-established UPR<sup>mt</sup> of invertebrates was not robustly induced in the mammalian muscle by mtDNA deletions but could still serve important functions upon different insults, for example in childhood-onset neurodegenerative mitochondrial disorders caused by direct disruption of OXPHOS subunits. This concept of different or alternative stress responses among mitochondrial (OXPHOS) diseases caused by different molecular mechanisms was highlighted in the biomarker study of this thesis (publication II). In our combined meta-analysis and retrospective measurement of serum FGF21 and GDF15 in patients and representative mouse models, we reported that the biomarkers were robustly induced upon primary or secondary defects of mtDNA translation but were not induced by isolated OXPHOS deficiency. These results on UPR<sup>mt</sup> and ISR<sup>mt</sup> highlight the need to specify the disease mechanisms and stress responses when studying the pathophysiology of mitochondrial diseases.

Together, results presented in this thesis offer comprehensive description of the pathophysiological events in mitochondrial myopathy. We describe conservation of the transcriptional and metabolic stress responses in mammalian systems with mtDNA maintenance or expression defects. Furthermore, we demonstrate sequential progression for the mammalian ISR<sup>mt</sup> with tissue-specific regulation of the metabolic pathology by FGF21. Our data on context-dependent stress responses unlock new directions for future research, and offer tools for diagnostics as well as follow-up and management of mitochondrial disease progression.



## ACKNOWLEDGEMENTS

This doctoral thesis was carried out in the University of Helsinki, the Faculty of Medicine, in Academy of Finland Centre of Excellence (FinMIT). The research was funded by general grants of the laboratory of Professor Anu Wartiovaara and financial support was received from Helsinki Biomedical Graduate Program, Biomedicum Helsinki foundation, Maud Kuistila Memorial foundation, Orion Research foundation, Oskar Öflund foundation, Paulo foundation, Petter and Margit Forsström foundation and Waldemar von Frekell foundation.

First, I wish to thank my supervisor, Professor Anu Wartiovaara. During the years in your laboratory I was given the opportunity to grow, and experience freedom to plan and execute numerous projects independently as well as in teams with talented people. You trusted me with responsibilities and collaborations I personally did not think I could handle – you know how to gently boost the lowest self-esteem.

I express my gratitude for Professor Kirsi Virtanen for being the official opponent of this thesis, and associate Professor Valeria Tiranti and assistant Professor Sjoerd Wanrooij for lending their time on revision of my thesis. Elina Ikonen and Henri Huttunen are thanked for the support and atmosphere in thesis follow-up meetings, and Elina Ikonen also for accepting the position as representative of the Faculty of Medicine in my thesis evaluation committee.

The mere number of authors in the original articles of this thesis is respectable. Therefore, I want to thank all the co-authors and collaborators for their important contribution for this thesis. I want to express my special gratitude to Liliya Euro, who dedicated countless hours to teach me the essentials and more. You have a gift to detect distress and never walk away. Joni Nikkanen, our symbiotic projects were the most educational and enjoyable of my PhD. I miss you daily. Jenni Lehtonen is warmly acknowledged for the seamless collaboration on our common paper. Alternately we carried each other and the project through three sequential maternity leaves. I want to thank Chris Jackson for his work to bring together the final block of this thesis. Chris Carroll, the amount of time and effort you dedicate to discussion and teaching is admirable. Thank you both Chris Carroll and Riikka Jokinen for language revision of my thesis.

I want to express my gratitude to the people in Turku PET-centre. This collaboration has shaped the form of my book more than any other experiment. Päivi Marjamäki, Heidi Liljenbäck and Anne Roivainen are true professionals who made me feel welcome on my countless visits during the years. Thank you Anu Harju, Markus Innilä and Tuula Manninen for help and expertise.

All the past and present members of our scientific society are thanked for the support and atmosphere. Thank you, fellow students and role models, for the past years. I am happy to call many of you my friends now, and in the future. I am grateful to be surrounded by all the wonderful friends outside work. You have listened, counseled and provided the wonderful distraction that is called life.

Finally, I wish to thank my family. My parents always trusted me to take responsibility, make my own decisions, and supported those. They told me I could do anything that I wanted. Well – I did this. Tuomas, without you, I would have failed or quit the difficult things in my life so many times I cannot even count. Yet, far too rarely I remember to give you the credit you deserve for the unconditional support and love. And thank you Aliisa, you are the best.

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